CHARLES UNIVERSITY
Faculty of Physical Education and Sport

Electrophysiological models of seizure like activity for NO detection in vitro

DIPLOMA THESIS

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Prague 2010

SUMMARY

Definition of work:
Electrophysiological models of seizure like activity for NO detection in vitro

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Aim: The aim of the thesis is to introduce nitric oxide (NO) and its role in physiology and pathophysiolgy of central nervous system (CNS), with the intention of epileptiform activity in the nervous tissue. Our research was realized on rat hippocampal slices *in* vitro and it compares the experimental biochemical models (low-Mg^{2+}, high-K^{+}, Carbachol, Bicuculline and 4-Aminopyridine model). These changes in the environment of slice should evoke the epileptiform activity in the nervous tissue of the rat. The final aim of the thesis is to create an experimental model of epileptiform activity in vitro in our setup for imaging techniques and NO detection.

Methods: Experiments were performed in rat hippocampal slices in vitro. Rats were deeply anaesthetized with ether and decapitated. Brain was rapidly removed from the scull, cut in oxygenated (95%) and cooled (0, 5 °C) artificial cerebrospinal fluid (ACSF) using vibratome in coronal slices of 400μm of thickness. They were submerged into recording chamber where ACSF in adequate concentration was perfused (5 ml/min and temperature ± 33 °C) using a peristaltic pump. Data recordings were made in the submerged type chamber. Synaptic activation of the tissue was made with bipolar stimulating electrode placed in hillus of the dentate gyrus of hippocampus (Mossy fibers). Detection electrode was placed in pyramidal layer of CA1 region. Single shock stimulation with supramaximal stimulus was applied and then other stimuli in determined values according to stimulation protocol. Experimental models were performed by changing the concentration of ions in the ACSF (low-Mg^{2+} and high-K^{+}

model) or by addition of chemical into this solution (Carbachol, Bicuculline and 4-AP model).

Results: We evaluated eight hippocampal slices with following results. We haven't been able to elicit tonic clonic seizures in our setup. The only model whose activity resembled to seizure activity was the high-K^+ model (10mM). With low-Mg^{2+} (zero Mg^{2+}), high-K^+ (10mM), Bicuculline (13μM and 33μM) in combination with high-K^+ (7mM KCl), 4-Aminopyridine (250μM), except for Carbachol treated slices, we were able to elicit epileptic discharges.

Key words: nitric oxide (NO), epilepsy, hippocampus, low-Mg^{2+}, high-K^+, Carbachol, Bicuculline, 4-AP.

SOUHRN

Název práce:
Elektrofyziologické modely epileptické aktivity pro detekci NO in vitro

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Cíl práce: Cílem této práce je představit oxid dusnatý (NO) a jeho roli ve fyziologii a patofyziologii centrálního nervového systému (CNS) se zaměřením na epileptickou aktivitu nervové tkáně. Náš výzkum, který byl proveden in vitro na potkaních hipokampálních řezech, porovnává experimentální biochemické modely (nízká koncentrace hořčíku Mg^{2+}, vysoká koncentrace draslíku K^{+}, Carbachol, Bicuculline a 4-aminopyridine model). Takto změněné podmínky prostředí řezu by měly vyvolávat epileptickou aktivitu v nervové tkáni potkana. Konečným cílem práce je vytvoření optimálního experimentálního modelu epileptické aktivity in vitro, který je předpokladem pro další experimenty především pro optické zobrazovací techniky, jejichž pomocí jsme schopni detekovat NO.

Metody: Měření bylo provedeno na řezech hippocampu laboratorních potkanů in vitro. Potkani byli dekapitováni v hluboké anestesii éterem. Mozek byl rychle exstirpován z lebky a nařezán v okysličeném (95%) a chlazeném (0,5 °C) uměle vytvořeném mozkomíšním moku s použitím vibratomu na frontální řezy o šířce 400 μm a uchovány po dobu nejméně 2h v ustalovací komůrce. Řezy byly poté přemístěny do nahrávací komůrky, kde byly zcela ponořeny do proudícího umělého mozkomíšního moku (5 ml/min a při teplotě ± 33 °C) a za použití peristaltického čerpadla. Stimulaci jsme prováděli bipolární stimulační elektrodou umístěné do oblasti hilus gyrus dentatus hippocampi. Detekční elektroda byla umístěna do pyramidové vrstvy v oblasti CA1. Experimentální modely byly vytvořeny změnou koncentrace iontů v uměle vytvořeném

mozkomíšním moku (u modelu s nízkou koncentrací hořčíku a vysokou koncentrací draslíku) nebo aplikací farmaka do tohoto roztoku (u modelu s použitím Carbacholu, Bicucullinu a 4-AP) .

Výsledky: Hodnotili jsme osm hippocampálních řezů s těmito výsledky. Nepodařilo se nám vyvolat tonicko-klonické záchvaty epileptické aktivity na žádném z řezů a u žádného experimentálního modelu. Jediný model, který se svým průběhem přiblížil epileptické aktivitě byl model s vysokou koncentrací draslíku (10mM). Kromě modelu s použitím Carbacholu, jsme úspěšně vyvolali epileptické výboje u nízké koncentrace Mg^{2+}, vysoké koncentrace K^{+} (10mM), Bicucullinu (13μM and 33μM) v kombinaci s vysokou koncentrací (7mM KCl) a 4-AP (250μM).

Klíčová slova: oxid dusnatý (NO), epilepsie, hippocampus, nízká koncentrace Mg^{2+}, vysoká koncentrace K^{+}, Carbachol, Bicuculline, 4-AP.

ACKNOWLEDGEMENTS

I would like to thank my supervisor of the thesis, MUDr. Jakub Otáhal Ph.D., and the consultant, Mgr. Renata Konopková, for their guidance, advice and for answering all of my questions during the research. Their knowledge, skills and experiences in research and science were essential for the completion of the work.

This research was carried out at the Academy of Science of the Czech Republic.

DECLARATION

I declare that I compiled this Diploma Thesis by myself and with using mentioned references.

Prohlašuji, že jsem tuto diplomovou práci zpracoval samostatně a s použitím uvedené literatury.

V Praze dne 10. 4. 2010 Karel Novák

CONTENTS

1. INTRODUCTION

Central nervous system is a leader, supervisor and coordinator of all processes in the human body. Thanks to CNS we are able to move, behave and live in the society with other people, all these complex functions are enabled by virtue of many biochemical processes that occur in molecular level. Neuron, electrically excitable neuronal cell is responsible for the transmission of the signal and neuronal communication in general. With increasing knowledge about processes in CNS and better equipment for the detection even in the cellular level we are discovering many new substances that can influence neuronal activity and help us to deal with different pathological states of the CNS. One of these discoveries is NO detection and determination its role in neuronal tissue. Having this gas as a signaling molecule, radically changed the whole concept of neural communication and posed many new questions that need to be elucidated.

Detection of this molecule poses some difficulties because of its gaseous characteristics. On the other hand, these characteristics bring some advantages because this small, uncharged and fat-soluble molecule can diffuse widely and readily enters the cells.

Control of the synthesis of NO is the key to regulating its activity and functional results. There are three known isoforms of NO synthases (NOS) and each of them has its specific function in human body; neuronal nNOS, endothelial eNOS and inducible iNOS.

NO seems to be mostly kept in mind as a smooth muscle relaxant with vasodilatation effect so that attached as a support in heart and cerebral stroke or penile erection where increasing a blood supply. But it is necessary to involve it into the functional context and realize that it has been related such an important processes like respiratory chain and Krebs cycle, running in mitochondria and processing the oxygen and generating the energy, essential for all organisms (Beltrán et al., 2000). Centrally released NO has been proposed in functions of CNS such a brain development, visual processing, discriminative learning, food and drink behaviour and many and many

others; but also in peripheral functions such a regulation of blood pressure, heart rate, gastric acid secretion, etc. It has been implicated in perception of pain when synthesis of NO enhances spinal facilitation of the afferent input whereas inhibition of NO can have antinociceptive effects. There is an evidence of overproduction of NO in cerebral ischemia caused by activation of nNOS and involved in neuronal damage but NO produced by eNOS in the same case play a protective role by maintaining regional cerebral blood flow. It is also related in numerous disorders of CNS and in the etiology of neurological conditions, including autoimmune and chronic neurodegenerative diseases. (Esplugues, 2002)

In the present studies, there is a tendency to investigate the role of NMDA/NOS system in epileptiform activity (Schuchmann, 2002). NMDA receptor is a specific type of ionotropic glutamate receptor. These receptors when tonically activated can trigger an excessive increase in intracellular calcium; nerve cells start to induce abnormal excessive activity and intracellular calcium accumulation or even get damaged, so called excitotoxicity. The activity of NO is dependent on the increased levels of intracellular Ca^{2+} and binding to calmodulin and that the connection between NMDA receptors and NO production.

NO has been proposed as the retrograde messenger which co-ordinates the enhancement of both pre- and post-synaptic mechanism involved in two forms of synaptic plasticity; namely long-term potentiation (LTP) and long-term depression (LTD) (Esplugues, 2002). NO, respectively NOS, as a proposed mediator of LTP, influences the enzymatic activity in mitochondria and the whole process of LTP where the connection between neuronal cells can get improved in their strength relates to NMDA receptors. When these receptors are stimulated simultaneously with the sufficient strength, intersynaptic transport modulates in increasing postsynaptic cell's sensitivity and finally leads into the enhancement of existing receptors' activity or even their amount. Then called the process of modulation, it underlies learning and memory as a major cellular mechanism; and when lasting longer, these changes in brain are thought to participate in construction of neuronal circuits during development and to relate to long-term formation of engrams.

This process of learning, modulation and synaptic plasticity reminds a work of physiotherapist who teaches the patient how to recover lost function of the movement or

reeducates him the new one. This work is not fixed on the muscle but the supervision, central nervous system. According to this statement there is no doubt that this treatment should be mostly aimed on the function of organ, not on its structure, in accordance with idiom of St. Hilaire "The function forms organ" and by the name of principles of neuroplasticity**.**

We know that NO is involved in many aspects of CNS function but we are still far away from the pharmacological breakthrough which could have a clinical impact in CNS released diseases. The experimental knowledge continues to grow and recent important findings, including the role of NO in CNS, help to raise a prospect of future therapeutic leads, thus achieving the pharmacological goal of linking physiological knowledge with drug development. This physiological knowledge will help not only to doctors in pharmacological treatment but also to other occupations in medicine, such as physiotherapist, ergotherapist, medical technicians etc. Because these clinicians do not work only in routine practice of hospitals and similar institutions, but they cooperate in researches of many clinical branches. Moreover, they are expected, as the graduated of Master's degree, to know all of the contexts of disorders which they treat and to use these in their treatment. They are also expected to be a part of educational system in clinical medicine and to hand on their experiences and skills to other students. All of that makes them an essential part of multidisciplinary team.

There are many investigations in progress in the laboratory conditions of Czech Academy of Science to determine the physiological and pathophysiological aspects of disorders of CNS such a cerebral stroke or epilepsy, on animal models both *in vitro* and *in vivo*. We are focused on the detection of NO in epileptiform activity. For this purpose we have to create a functional and optimal model of these seizures, applicable in our setup, first of all *in vitro*, on rat hippocampal slices which are passed for the most suitable tissue for this research because of its unidirectional connectivity, similarity to the human structure and very well known morphology.

Success in these models *in vitro* anticipates following investigations *in vivo* and imaging techniques; for detection and determination the role of nitric oxide, possibly miraculous signaling molecule, in CNS.

2. THEORETICAL BACKROUND

2.1 ANATOMY

2.1.1 NEURON AND GLIA

Neuron is a nerve cell. It is an electrically excitable cell that processes and transmits information by electrochemical signaling. (Levitan & Kaczmarek, 1997)

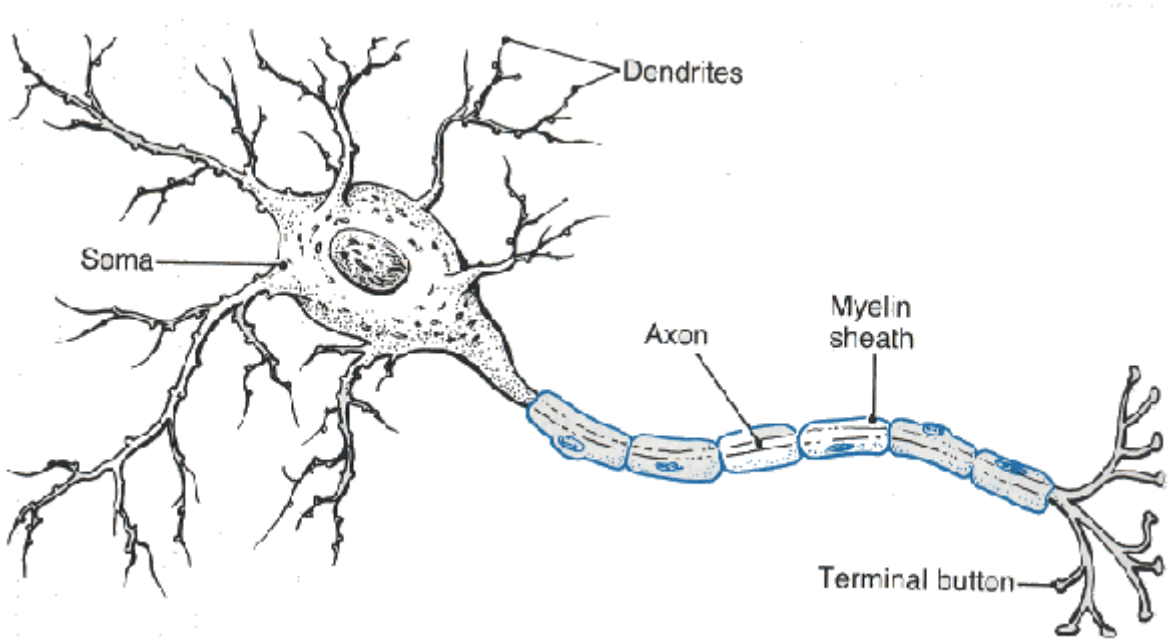


Figure 2.1: Neuron and its structure (Image 1)

Glia is non-neuronal cell that maintain homeostasis, forms myelin, and provides support and protection for the brain's neurons. (Azevedo et al., 2009)

Some glial cells function primarily as the physical support for neurons. Others regulate the internal environment of the brain, especially the fluid surrounding neurons and their synapses, and nourish neurons. During early embryogenesis, glial cells direct

the migration of neurons and produce molecules that modify the growth of axons and dendrites. Recent research indicates that glial cells of the hippocampus and cerebellum participate in synaptic transmission, regulate the clearance of neurotransmitters from the synaptic cleft, release factors such as ATP, which modulate presynaptic function, and even release neurotransmitters themselves.

Traditionally glia has been considered to lack certain features of neurons. For example, glial cells were not believed to have chemical synapses (see Chapter 2.1.3.6) or to release neurotransmitters (see Chapter 2.3). They were considered to be the passive bystanders of neural transmission. However, recent studies have shown this to be untrue. For example, astrocytes are crucial in clearance of neurotransmitter from within the synaptic cleft, which provides distinction between arrival of action potentials (see Chapter 2.2.1.3.) and prevents toxic build up of certain neurotransmitters such as glutamate (excitotoxicity). Furthermore, at least in vitro, astrocytes can release neurotransmitter glutamate in response to certain stimulation.

The only notable differences between neurons and glial cells are neurons' possession of axons and dendrites, and capacity to generate action potentials.

Glia ought not to be regarded as 'glue' in the nervous system as the name implies; rather, it is more of a partner to neurons. They are also crucial in the development of the nervous system and in processes such as synaptic activity and synaptogenesis. Glia has a role in the regulation of repair of neurons after injury. In the CNS, glia suppresses repair. Glial cells known as astrocytes enlarge and proliferate to form a scar and produce inhibitory molecules that inhibit regrowth of a damaged or severed axon. (Levitan & Kaczmarek, 1997)

Shortly, there are a number of functional roles ascribed to glia:

- myelination
- act as a scaffolding for neuronal migration and axon outgrowth
- participate in the uptake and metabolism of the neurotransmitters that neurons use for intercellular communication
- take up and buffer ions from the extracellular environment
- act as scavengers to remove debris produced by dying neurons

Although the evidence is less definitive, glia also may:

- segregate groups of neurons one from another, and act as a electrical insulators between neurons
- provide structural support for neurons, fulfilling a role played by connective tissue cells in other organs
- play a nurturing role, supplying metabolic components and even proteins necessary for neuronal functions
- play a role in information handling and memory storage

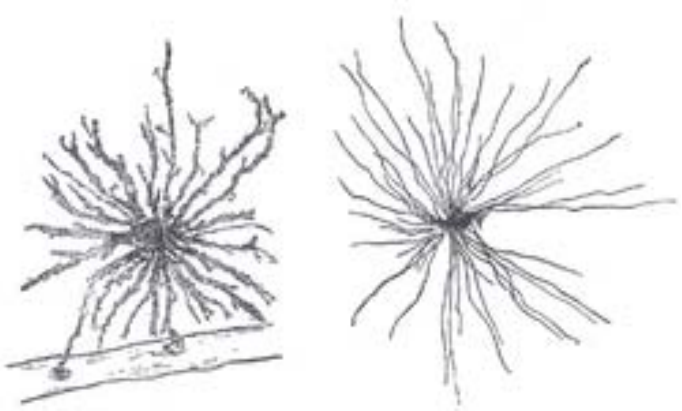


Figure 2.2: Glial cells (Image 2)

2.1.2 TYPES OF GLIAL CELLS IN CENTRAL NERVOUS SYSTEM

Glial cells can themselves be divided into several subclasses based on their appearance in the microscope. We have more types of glial cells surrounding neurons, microglial and macroglial cells. In the CNS, there are two main types of glial cells, **astrocytes** and **oligodendrocytes**. (Pelvig et al., 2008)

2.1.2.1 ASTROCYTES

The most abundant type of macroglial cell, astrocytes (also called astroglia), have numerous projections that anchor neurons to their blood supply. They regulate the

external chemical environment of neurons by removing excess ions, notably potassium, and recycling neurotransmitters released during synaptic transmission (Pelvig et al., 2008). Astrocytes are believed to play an important role in the volume changes of the nervous tissue a key component of changing optical properties (Konopková, 2004).

2.1.2.2 OLIGODENTROCYTES

Oligodendrocytes are cells that coat axons in CNS with their cell membrane forming a specialized membrane differentiation called myelin, producing the so-called myelin sheath. The myelin sheath provides insulation to the axon that allows electrical signals to propagate more efficiently. (Pelvig et al., 2008)

2.1.2.3 EPENDYMAL CELLS

Ependymal cells, also named ependymocytes, line the cavities of the CNS and make up the walls of the ventricles. These cells create and secrete cerebrospinal fluid (CSF) and beat their cilia to help circulate CSF. (Pelvig et al., 2008)

2.1.2.4 RADIAL GLIA

Radial glia cells arise from neuroepithelial cells after the onset of neurogenesis. Their differentiation abilities are more restricted than those of neuroepithelial cells. In the developing nervous system, radial glias function both as neuronal progenitors and as a scaffold upon which newborn neurons migrate. (Pelvig et al., 2008)

2.1.3 NEURON´S STRUCTURE

Neurons have structures and organelles that are in common with other cells. The most prominent organelle in the cell body is the **nucleus**, which contains the genetic material, DNA. The entire neuron is enclosed by a **plasma membrane**. This is a double layer of phospholipid molecules, which acts as a barrier preventing the contents of the cell from mixing with those of the extracellular space. It is also an effective electrical insulator, hindering the diffusion of charged ions in and out of the cell. Signaling in

nerve cells requires the controlled movement of ions across the plasma membrane, a process mediated by specialized proteins located in the membrane. The cell body contains **mitochondria** (see Chapter 2.1.3.1) to supply the cell's energy needs. In fact, because a great deal of energy is required to maintain the transmembrane ionic gradients that are essential for neuronal signaling, neurons tend to be particularly rich in mitochondria. There are also **ribosomes**, which are responsible for the synthesis of proteins destined for insertion into membranes, or for secretion; they are located on the membranous sacs of the **rough endoplasmatic reticulum**. Other membranous components include the **smooth endoplasmatic reticulum** and the **Golgi complex**, involved in the processing of proteins for membrane insertion or secretion, and **lysosomes** and other granules involved in the break-down and disposal of cellular components.

2.1.3.1 MITOCHONDRIA

Mitochondria are sometimes described as "cellular power plants" because they generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy. In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth.

This organelle is composed of compartments that carry out specialized functions. These compartments include the **outer membrane**, the **intermembrane space**, the **inner membrane**, and the **cristae** and **matrix**.

The most prominent roles of mitochondria are to **produce energy** in form of **ATP** (i.e., phosphorylation of ADP) through respiration, and to **regulate cellular metabolism**. The central set of reactions involved in ATP production is collectively known as the citric acid cycle, or the **Krebs Cycle**; and **respiratory chain** (Bruce et al., 1994)

But there are three structural elements unique to neurons, the **axon** (see Chapter 2.1.3.2), which is specialized for intracellular information transport, the **dendrite** (see Chapter 2.1.3.3), which is often the site at which information is received from other neurons, and the most highly specialized structure of all, the **synapse** (see Chapter

2.1.3.4), which is the point of information transfer between neurons (see Fig. 1.1). (Levitan & Kaczmarek, 1997)

2.1.3.2 AXONS

Axon is a long, slender projection of neuron that conducts electrical impulses away from the neuron's cell body. It originates at a cone-shaped thickening on the cell body called the axon hillock. Axon's structure is formed and maintained by the cytoskeleton. (Levitan & Kaczmarek, 1997)

2.1.3.3 DENDRITES

Dendrites are neuronal processes that tend to be thicker and much to be shorter than axons and they are often highly branched, giving rise to a dense network of processes known as the dendritic tree. There is a presence of numerous finger-like projections or thickenings on the dendrites of some neurons. These projections, called dendritic spines, arise from the main shaft of the dendrite. These spines are the synaptic input sites at which the neuron receives information from another cell. However, the role of the dendrite is not exclusively the receipt of information. Some dendrites share with axons the ability to transmit electrical signals, and in many nerve cells both information input and output occur on the same set of dendrite-like fine processes. (Levitan & Kaczmarek, 1997)

2.1.3.4 SYNAPSES

Intercellular communication means the passage of information from one part of the nervous system to another and it is the essence of nervous system function. This information transfer distinguishes the brain from other organs and the synapse carries out this task (Levitan & Kaczmarek, 1997). The adult human brain is estimated to contain from 10^{14} to 5×10^{14} (100-500 trillion) synapses. Every cubic millimeter of cerebral cortex contains roughly a billion of them (Alonso-Nanclares, 2008).

At a synapse, the plasma membrane of the signal-passing neuron (the **presynaptic** neuron) comes into close apposition with the membrane of the target

(**postsynaptic**) cell. Both the presynaptic and postsynaptic sites contain extensive arrays of molecular machinery that link the two membranes together and carry out the signaling process. (Rapport, 2005)

There are two different types of synapse:

Electrical synapse. The presynaptic and postsynaptic cell membranes are connected by channels that are capable of passing electrical current, causing voltage changes in the presynaptic cell to induce voltage changes in the postsynaptic cell. (Rapport, 2005)

Chemical synapse. The presynaptic neuron releases a chemical called a neurotransmitter that binds to receptors located in the postsynaptic cell, usually embedded in the plasma membrane. Binding of the neurotransmitter to a receptor can affect the postsynaptic cell in a wide variety of ways. (Rapport, 2005)

2.1.3.5 ELECTRICAL SYNAPSE

Signals can move in both directions through electrical synapses, it is bidirectional information transfer. Between the membranes of the pre- and postsynaptic cells are found **gap junctions**, cell-to-cell pores that allow ions and small molecules to pass freely from the cytoplasm of one cell to the next. This movement mediates intercellular signaling in electrical synapses. (Levitan & Kaczmarek, 1997)

Compared to chemical synapses, electrical synapses conduct nerve impulses faster, but unlike chemical synapses they do not have gain (the signal in the post synaptic neuron is always smaller than that of the originating neuron).

The response is always the same sign as the source. For example, depolarization (see Chapter 2.2.1.3) of the pre-synaptic membrane will always induce a depolarization in the post-synaptic membrane, and vice versa for hyperpolarization. (Purves et al., 2008)

2.1.3.6 CHEMICAL SYNAPSE

The presynaptic ending is swelling of the axon terminal, containing mitochondria and, the most important, a variety of vesicular structures. The pre- and

postsynaptic elements are separated by a gap, the **synaptic cleft** (Levitan & Kaczmarek, 1997). Synaptic vesicles are docked at the presynaptic plasma membrane at regions called active zones. Opposite is a region of the postsynaptic cell containing neurotransmitter receptors. Most synapses connect axons to dendrites, but there are also other types of connections, including axon-to-cell-body, axon-to-axon, and dendrite-to-dendrite (Weiss et al., 1994).

Chemical synapses are unidirectional. It means that rapid transfer of information only occurs from the pre- to postsynaptic cell. There is a delay that may be a millisecond or longer between the arrival of information at the presynaptic terminal and its transfer to the postsynaptic cell. (Levitan & Kaczmarek, 1997)

2.1.4 HIPPOCAMPUS

2.1.4.1 HUMAN

The **hippocampus** is a major component of the brains of humans and other mammals. It belongs to the **limbic system** and plays important roles in **long term memory and spatial navigation**. There is evidence that the hippocampus plays a role in finding shortcuts and new routes between familiar places. Like the cerebral cortex, with which it is closely associated, it is a paired structure, with mirror-image halves in the left and right sides of the brain. (Amaral & Lavemex, 2006)

Hippocampus processes information from cerebral cortex and limbic system and then it transfer information into anterior thalamus, hypothalamus and other parts of brain. Most of these information return to cerebral cortex. This rhythmic excitatory activity is a base of complex integrative processes as loading information into the long-term memory.

In Alzheimer's disease, the hippocampus is one of the first regions of the brain to suffer damage; memory problems and disorientation appear among the first symptoms. Damage to the hippocampus can also result from oxygen starvation (hypoxia), encephalitis, or medial temporal lobe epilepsy. (Best & White, 1999)

2.1.4.2 RAT

Hippocampal formation is a useful model for the study of properties of central nervous system because of its unidirectional connectivity, similarity to the human structure and very well known morphology. (Amaral & Witter, 1995)

Hippocampal formation of the rat with its surface area approximately 1.2cm^2 (the surface of the entire isocortex is estimated with 1.5cm^2) is a prominent component of the rat nervous system and attracts the attention of neuroanatomists since the beginning of formal study of the nervous system. (Amaral & Witter, 1995)

The hippocampus as a whole has the shape of a curved tube, which has been analogized variously to a seahorse, a ram's horn (Cornu Ammonis - Ammons Horn - CA). It consists of ventral and dorsal portions, both of which share similar composition but are parts of different neural circuits. This general layout holds across the full range of mammalian species, from hedgehog to human, although the details vary. (Amaral & Lavemex, 2006)

The hippocampal formation includes the **entorhinal cortex (EC)**; **dentate gyrus (DG)**; **hippocampus proper**, which is subdivided into fields CA1, CA2, and CA3 (although the existence of CA2 in the rat is controversial and rarely mentioned in the physiology literature); and the **subicular complex**, consisting of the subiculum, presubiculum, and parasubiculum. (Amaral & Witter, 1995)

Dentate gyrus (DG) contains three layers, **molecular layer** (contains dendrites of granule cells, basket and polymorphic cell, axonal arbors); the **granule cell layer (principal cell layer)** which is made up primarily of densely packed columnar stacks of granule cells; **the polymorphic cell layer (mossy cells)**. (Amaral & Witter, 1995)

Many neurons in the rat and mouse hippocampus respond as place cells: that is, they fire bursts of action potentials when the animal passes through a specific part of its environment. Hippocampal place cells interact extensively with head direction cells, whose activity acts as an inertial compass, and with grid cells in the neighboring entorhinal cortex.

2.1.4.3 TRISYNAPTIC CIRCUIT

The perforant path-to-dentate gyrus-to-CA3-to-CA1 was called the trisynaptic circuit by Per Andersen, who noted that thin slices could be cut out of the hippocampus perpendicular to its long axis, in a way that preserves all of these connections. This observation was the basis of his lamellar hypothesis, which proposed that the hippocampus can be thought of as a series of parallel strips, operating in a functionally independent way. (Andersen et al., 1971)

The hippocampus forms a principally unidirectional network with input from the entorhinal cortex (EC) that forms connections with the dentate gyrus (DG) and CA3 pyramidal neurons via the Mossy fibers (MF). They send axons to CA1 pyramidal cells via the Schaffer collateral pathway (SC), as well as to CA1 cells in the contralateral hippocampus via the associational commissural pathways (AC). CA1 neurons also receive input directly from the perforant path and send axons to the Subiculum (Sb). These neurons in turn send the main hippocampal output back to the EC, forming a loop.

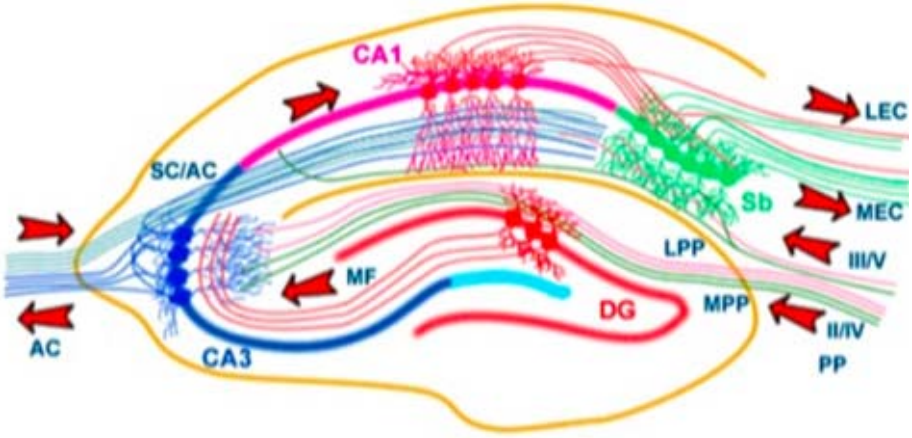


Figure 2.3: Trisynaptic circuit (Image 3)
(AC = associational commissural pathways; EC = entorhinal cortex; DG = dentate gyrus; PP = perforant pathway - medial or lateral; MF = Mossy fibers; SC = Schaffer collaterals)

2.2 ELECTROPHYSIOLOGY

2.2.1 ELECTRICAL SIGNALING IN NEURONS

Neurons, like all other cells, exhibit a voltage difference known as the **membrane potential** across their plasma membranes. This potential results from the unequal distribution of electrical charge, carried by ions (potassium, sodium, calcium and chloride), on two sides of the membrane. Some ions can move across the plasma membrane more readily than the others and such differential permeability arises because of the presence of specialized proteins known as **ion channels.** (Levitan & Kaczmarek, 1997)

2.2.1.1 ION CHANNELS

Ion channels are integral membrane proteins with a pore through which ions can travel between extracellular and intracellular space. Most channels are specific (selective) for one ion, especially potassium and sodium channels are very selective for their proteins. Ion channels may have different states (corresponding to different conformations of the protein), but each state is either open or closed. They can be classified by the nature of their gating, so that by their response to the environment; **voltage-gated channels** and **ligand-gated channels**.

Voltage-gated channels open and close in response to the voltage across the membrane, they have a crucial role in excitable neuronal tissue, allowing a rapid and coordinated depolarization. For example, voltage-gated calcium channels play an important role in neurotransmitter release in pre-synaptic nerve endings. Ligand-gated channels form another important class, opened and closed in response to the binding of a ligand molecule, such as neurotransmitter. Such receptors located at synapses convert the chemical signal of presynaptically released neurotransmitter directly and very quickly into a postsynaptic electrical signal.

2.2.1.2 RESTING POTENTIAL

The resting potential is usually in the range –40 to –90mV, in most neurons of human body it balanced -70 to -90mV. This negative membrane potential indicates that the inside of the cell membrane is more negative than the outside. When the membrane potential is less negative than the resting potential, the cell is said to be **depolarized**; when it is more negative, the cell is **hyperpolarized**. (Levitan & Kaczmarek, 1997)

The cell membrane prevents charged particles such as mentioned ions from freely diffusing into and out of the cell. There are two basics ways that they can get in or out. The first is with **passive transport**. Basically the cell has a protein, called ion channels and mentioned above, in the cell membrane that it can open and close like a water faucet. It is specific for certain kinds of chemicals like these ions. When it opens, then the ions can flow down their gradient from the more concentrated area to the less concentrated area. The other way to get ions in or out of cells is to by **active transport**. The cell uses some of its own energy, represented by ATP, to actively pump the chemicals against their gradient. The neuron has an ion pump, **Na^+/K^+-ATPase** also called **sodium-potassium pump**, that actively pumps three Na^+ ions out and takes in two K^+ ions. This means that a net positive charge flows out of the neuron. This is what gives the cell its negative potential.

2.2.1.3 ACTION POTENTIAL

An action potential (AP) is a short-lasting event in which the electrical membrane potential of a cell rapidly rises and falls, following a stereotyped trajectory. It plays a central role in cell-to-cell communication. (Barnett & Larkman, 2007)

As the depolarizing stimulus gets larger, a critical stimulus strength or **threshold** is reached. This active response of the membrane when the depolarization exceeds threshold is the nerve impulse or **action potential**. The threshold is essential to ensure that small random depolarization of the membrane do not generate APs. Only stimuli of sufficient importance (reflected by their larger amplitude); result in information transfer via AP. Another important property of APs is that they are all-or-none events; this **all-or-none law** is an essential feature of axonal signaling transmission. Once the stimulus is above threshold, the amplitude of the response no longer reflects the amplitude of the

stimulus. It means that information about stimulus strength must be represented, encoded, in the axon in some way other than action potential amplitude**;** the amplitude of the AP is generally independent of stimulus intensity. The **latency**, the time delay from the onset of the stimulus to the peak of the AP, is a function of stimulus strength. **The stronger stimulus the shorter delay between stimulus and AP**. For several milliseconds after the **firing** of AP, it is impossible to evoke another AP no matter how large the depolarizing stimulus. The axon is refractory to stimuli during this time, so it is called **refractory period.** This absolute refractory period is followed by a relative refractory period, during which the stimulus must be larger than normal to evoke an AP. During the absolute refractory period the threshold is essentially infinite, and no stimulus, no matter how large, can exceed it. During the relative refractory period the threshold is larger than normal, that is, it requires a larger than normal stimulus to exceed it (Levitan & Kaczmarek, 1997). In summary we can say about electrical signaling in neurons that:

- there is a **threshold** for generation of AP that guarantees that small random variations in the membrane potential are not misinterpreted as meaningful information;
- the **all-or-none law** guarantees that once an AP is generated, it is always full size, minimizing the possibility that information will be lost along the way;
- the **strength-latency relationship** and the **refractory period**, together with the threshold, allow the encoding of information in the form of a **frequency** code; and
- the phenomenon of **passive spread**, which arises simply from the cable-like properties of the axonal membrane, allows the propagation of action potentials along the axon and the transfer of information over long distance within the neuron.

The course of the AP can be divided into five parts: the **rising phase**, the **peak phase**, the **falling phase**, the **undershoot phase**, and finally the **refractory period**. During the rising phase the membrane potential depolarizes (becomes more positive), it is also called **depolarization**. The point at which depolarization stops is called the peak phase. At this stage, the membrane potential reaches a maximum. Subsequent to this, there is a falling phase. During this stage the membrane potential hyperpolarizes

(becomes more negative), also called **repolarization**. When repolarization is about 70% completed, the rate of repolarization decreases and the curve approaches the resting level more slowly. The sharp rise and rapid fall are the spike potential of the neuron, and the slower fall at the end of the process is the **after-depolarization**. The undershoot phase, also called **hyperpolarization**, is the point during which the membrane potential becomes temporarily more negatively charged than when at rest. Finally, the time during which a subsequent action potential is impossible or difficult to fire is called the refractory period, also called **after-hyperpolarization**, which may overlap with the other phases (see Fig. 2.4). (Purves et al., 2008)

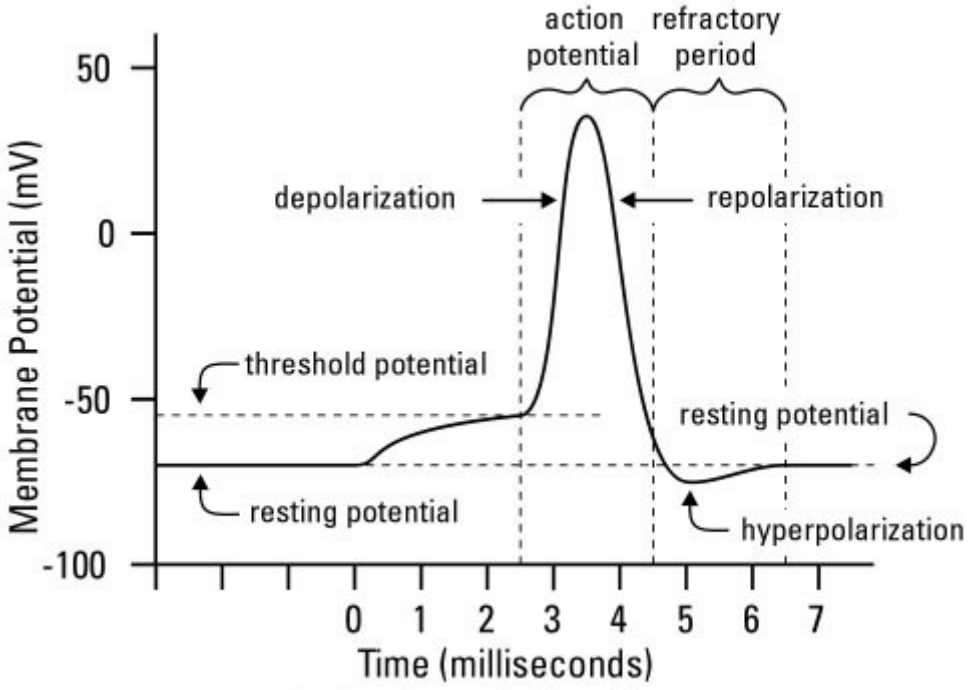


Figure 2.4: Action potential and its phases (Image 4)

Ions are also responsible for the initiation or transmission of AP in the neurons, like in the case of resting potential. Ion flow in and out of the cell becomes in few steps (see Fig. 2.5):

1. The first step of AP is that the Na^+ channels open allowing a flood of sodium ions into the cell. This causes the membrane potential to become positive.

2. At some positive membrane potential the K^+ channels open allowing the potassium ions to flow out of the cell.
3. Next the Na^+ channels close. This stops inflow of positive charge. But since the K^+ channels are still open it allows the outflow of positive charge so that the membrane potential plunges.
4. When the membrane potential begins reaching its resting state the K^+, channels close.
5. Now the sodium-potassium pump does its work and starts transporting sodium out of the cell, and potassium into the cell so that it is ready for the next AP.

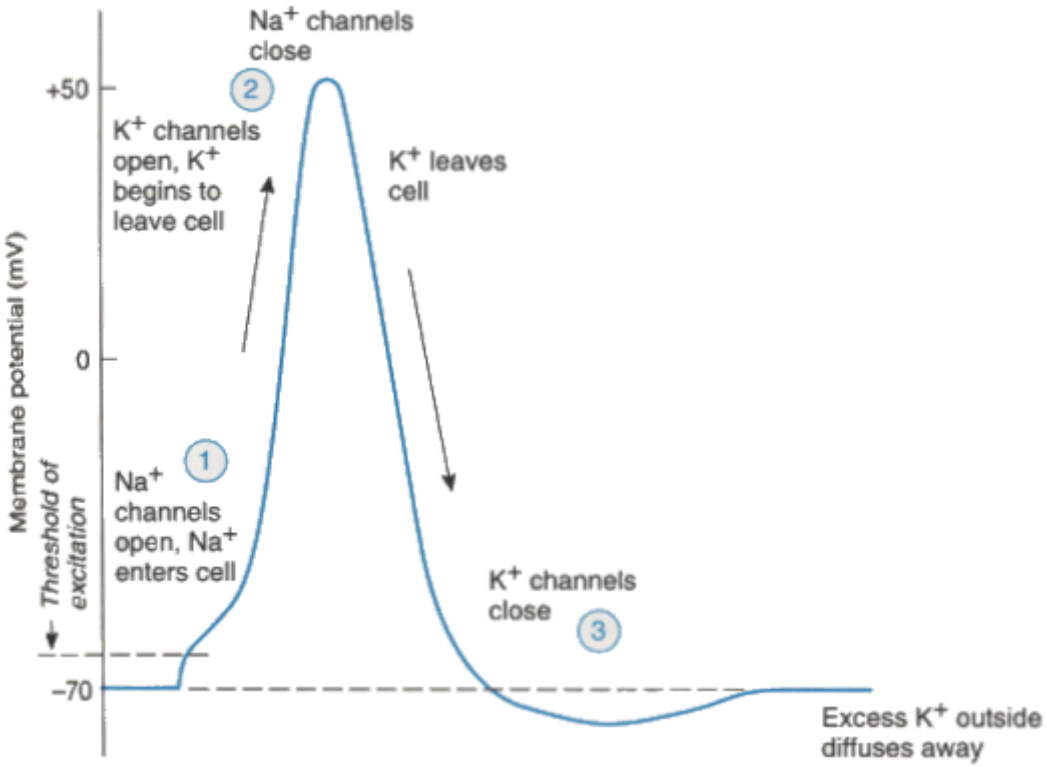


Figure 2.5: Action potential and ion flow (Image 5)

2.3 NEUROTRANSMITTERS IN CNS

Neurotransmitters are endogenous chemicals, which relay, amplify, and modulate signals between a neuron and another cell. Major neurotransmitters in CNS include amino acids such as glutamate (see Chapter 2.3.4), aspartate, serine, γ-aminobutyric acid (GABA) (see chapter 2.3.3), glycine; monoamines such as dopamine, noradrenaline, adrenaline, histamine, serotonin, melatonin; and many others like an acetylcholine, over 50 neuroactive peptides, single ions, such as synaptically released zinc and a few gaseous molecules such as nitric oxide (NO) and carbon monoxide (CO).

Description of all neurotransmitters is over the range of this thesis so for their connectivity in epileptiform seizures and research; and a role in function and synthesis of NO in CNS (see Chapter 2.4.3.), following list include two major representatives; inhibitory GABA and excitatory glutamate.

2.3.1 NEUROTRANSMITTER RELEASE AND RECEPTOR BINDING

The process begins with a wave of electrochemical excitation called an action potential traveling along the membrane of the presynaptic cell, until it reaches the synapse. The electrical depolarization of the membrane at the synapse causes channels to open that are permeable to calcium ions. Calcium ions then trigger a biochemical cascade that results in vesicles fusing with the presynaptic membrane and releasing their contents to the synaptic cleft within 180μsec of calcium entry. (Llinás et al., 1981)

Receptors on the opposite side of the synaptic gap bind neurotransmitter molecules and respond by opening nearby ion channels in the postsynaptic cell membrane, causing ions to rush in or out and changing the local transmembrane potential of the cell. The resulting change in voltage is called a postsynaptic potential. In general, the result is **excitatory**, in the case of depolarizing currents, or **inhibitory** in the case of hyperpolarizing currents. Whether a synapse is excitatory or inhibitory depends on what type(s) of ion channel conduct the postsynaptic current display(s),

which in turn is a function of the type of receptors and neurotransmitter employed at the synapse. (Llinás et al., 1981)

2.3.2 AGONIST AND ANTAGONIST

Receptors can be activated or inactivated by either endogenous (such as hormones and neurotransmitters) or exogenous (such as drugs) agonists and antagonists, resulting in stimulating or inhibiting a biological response.

An **agonist** is a chemical that binds to a receptor of a cell and triggers a response by the cell. An agonist often mimics the action of a naturally occurring substance.

A receptor **antagonist** is a type of receptor ligand or drug that does not provoke a biological response itself upon binding to a receptor, but blocks or reduces agonist-mediated responses. In pharmacology, antagonists have affinity but no efficacy for their cognate receptors, and binding will disrupt the interaction and inhibit the function of an agonist or inverse agonist at receptors. (Kenakin, 2006)

2.3.3 GABA

GABA (γ-aminobutyric acid) is the chief **inhibitory neurotransmitter** in the central nervous system. It acts at inhibitory synapses in the brain by binding to specific transmembrane receptors in the plasma membrane of both pre- and postsynaptic neuronal processes. This binding causes the opening of ion channels to allow the flow of either negatively charged chloride ions into the cell or positively charged potassium ions out of the cell. This action results in a negative change in the transmembrane potential, usually causing hyperpolarization. Two general classes of GABA receptor are known: $GABA_A$ in which the receptor is part of a ligand-gated ion channel complex, and $GABA_B$ metabotropic receptors, which are G protein-coupled receptors that open or close ion channels via intermediaries (G proteins). Neurons that produce GABA as their output are called GABAergic neurons.

$GABA_A$ receptors are chloride channels. It means that when activated by GABA, they allow the flow of chloride ions across the membrane of the cell. Whether

this chloride flow is excitatory - depolarizing (makes the voltage across the cell's membrane less negative), shunting (has no effect on the cell's membrane) or inhibitory - hyperpolarizing (makes the cell's membrane more negative) depends on the direction of the flow of chloride. When net chloride flows out of the cell, GABA is excitatory or depolarizing; when the net chloride flows into the cell, GABA is inhibitory or hyperpolarizing. When the net flow of chloride is close to zero, the action of GABA is shunting. (Watanabe et. al., 2002)

Drugs that act as agonists of GABA receptors (known as **GABAergic drugs**) or increase the available amount of GABA typically have relaxing, anti-anxiety and anti-convulsive effects. Antagonists of GABA, such as Bicuculline (see Chapter 2.7.4.7) play an opposite role and block their inhibitory action.

2.3.4 GLUTAMATE

Glutamate is a non-essential amino acid. It is the most abundant **excitatory neurotransmitter** in the nervous system. At chemical synapses, glutamate is stored in vesicles. Nerve impulses trigger release of glutamate from the pre-synaptic cell. In the opposing post-synaptic cell, glutamate receptors, such as the NMDA receptors (see Chapter 2.3.5.), bind glutamate and are activated. Because of its role in synaptic plasticity, glutamate is involved in cognitive functions like learning and memory in the brain, this process is called neuroplasticity (see Chapter 2.6). (McEntee & Crook, 1993) Glutamate is also responsible for PDS (see Chapter 2.7.5) behaviour that is recorded intracellulary in association with epileptic discharges.

Glutamate receptors can be divided into two groups according to the mechanism by which their activation gives rise to a postsynaptic current. **Ionotropic** glutamate receptors (NMDA, AMPA and kainate receptor) form the ion channel pore that activates when glutamate binds to the receptor. **Metabotropic** glutamate receptors (mGluR) indirectly activate ion-channels on the plasma membrane through a signaling cascade that involves G proteins. Ionotropic receptors tend to be quicker in relaying information but metabotropic are associated with a more prolonged stimulus. (Palmada & Centelles, 1998)

2.3.5 NMDA RECEPTORS

NMDA receptor is a specific type of ionotropic glutamate receptor. NMDA (N-methyl D-aspartate) is the name of an agonist that binds to NMDA receptors but not to other glutamate receptors. Activation of NMDA receptors results in the opening of an ion channel that is nonselective to cations. A unique property of the NMDA receptor is its voltage-dependent activation, a result of ion channel block by extracellular Mg^{2+} ions. This allows voltage-dependent flow of Na^{+} and small amounts of Ca^{2+} ions into the cell and K^{+} out of the cell. Calcium flux through NMDA receptors is thought to play a critical role in synaptic plasticity (see Chapter 2.6), a cellular mechanism for learning and memory.

With regard to synaptic plasticity, the role of the NMDA receptor is best described as coincidence detection. It means that only if both the pre- and postsynaptic cells are simultaneously active will NMDA receptors become unblocked and allow calcium ions to enter the postsynaptic cell. Thus, the NMDA receptor converts an electrical signal into a biochemical signal that can trigger synaptic plasticity.

NMDA receptors are modulated by a number of endogenous and exogenous compounds and play a key role in a wide range of physiologic and pathologic processes, such as excitotoxicity, pathological process where nerve cells are damaged and killed by glutamate and similar substances, so they play an important role in neurological disorders such as stroke, traumatic brain injury or epilepsy. (Liu & Zhang, 2000)

2.4 NITRIC OXIDE

Nitric oxide (NO) is a gaseous free radical synthesized from L-arginine in several mammalian cells by the enzyme nitric oxide synthase (NOS) and is known as a neuronal messenger. (Schuchmann et al., 2002)

The physical properties of NO prevent its storage in lipid-lined vesicles and metabolism by hydrolytic depredatory enzymes. NO is synthesized on demand and is neither a stored in synaptic vesicle nor release by exocytose, but simply diffuses from nerve terminals. (Esplugues, 2002)

2.4.1 HISTORY

The discovery of the biological functions of NO in the 1980s came as a complete surprise and caused quite a stir. NOS was first identified and described in 1989, its three major isoforms were cloned and purified between 1991 and 1994, and in 1994 there were several reviews of the work on these enzymes until this time. Then “NO and NOS boom” started in publishing and research, including a great deal on the structure, function and inhibition of the enzymes. Nitric oxide was named "Molecule of the Year" in 1992 by the journal Science, a Nitric Oxide Society was founded, and a scientific journal devoted entirely to nitric oxide was established.

The first X-ray crystal structures of NOS domains have been presented and published in 1998 and 1999; and the importance of the field of nitric oxide research was recognized in 1998 by the award of the Nobel Prize to R. Furchgott, L. Ignaro and F. Murad for the work that led to the discovery of NO as a biological mediator produced by mammalian cells. (Alderton et al., 2001)

It is estimated that yearly about 3,000 scientific articles are published on the biological roles of NO what makes this molecule one of the most discussed theme in physiology, pharmacology and other research branches.

2.4.2 NITRIC OXIDE SYNTHASE (NOS)

Control of the synthesis of NO is the key to regulating its activity. There are three known isoforms of NOS and each of them has its specific function in human body. Endothelial NOS (**eNOS**) and inducible NOS (**iNOS**) are present in the nervous system. Neuronal NOS (**nNOS**) is the principal isoform present in said system (Esplugues, 2002). Some authors (Lamas & Cadenas, 2006) describe the fourth type of this enzyme, so extended of mitochondrial NOS (mtNOS) as a subtype of nNOS which has an important role in intracellular signaling and apoptosis, cellular death.

nNOS and eNOS are the constitutive calcium-dependent and produce low levels of gas as a cell signaling molecule, whereas iNOS is calcium-independent and produces large amounts of gas which can be cytotoxic. (Esplugues, 2002)

eNOS is originally found in the endothelial cells that line the lumen of blood vessels and the specific function of NO synthesized by this enzyme is vasodilatation. iNOS is originally found in macrophages, a cell acting in both non-specific and specific defense, and its function is specialized in immune defense against pathogens. Whereas the levels of eNOS and nNOS are relatively steady, expression of iNOS genes awaits an appropriate stimulus (e. g., ingestion of a parasite). nNOS produces NO in nervous tissue in both the central and peripheral nervous system. It performs a role in cell communication and is associated with plasma membranes. All types of NOS produce NO from L-arginine; α-amino acid ideal for binding negatively charged groups as proteins; with the aid of molecular oxygen and NADPH (α-nicotinamide adenine dinucleotide phosphate) which provides the reducing equivalents for biosynthetic reactions and anabolic pathways, such as lipid synthesis, cholesterol synthesis or glucose synthesis.

nNOS-containing neurons are presented in many areas of the CNS with the highest densities occurring in the accessory olfactory bulb and granule cells of the cerebellum. nNOS can be located either pre- or post-synaptically and is particularly implicated in neural signaling, neurotoxicity, synaptic plasticity and modulation of behavioral pathways such as learning or expression of pain. (Esplugues, 2002) eNOS is mainly involved in the regulation of vascular function and, although also present in

some populations of neurons (Dinerman et al., 1994) and glia (Wiencken & Casagrande, 1999), is predominantly located in the endothelial cells of cerebral vessels. Finally, induction of iNOS in glial cells is implicated in the unspecific immune response of the brain and is usually associated with pathological conditions (Murphy, 2000).

The aim of this thesis is to introduce a role of NO in CNS, respectively the research in vitro in the nervous tissue, so the text below is also aimed on all relative in this tissue, so that mostly in nNOS. Description of other forms of NOS and produced NO by themselves rounds off the idea about the functioning of CNS as a complex, including vascular, immune and other support for its function.

2.4.3 REGULATION OF nNOS

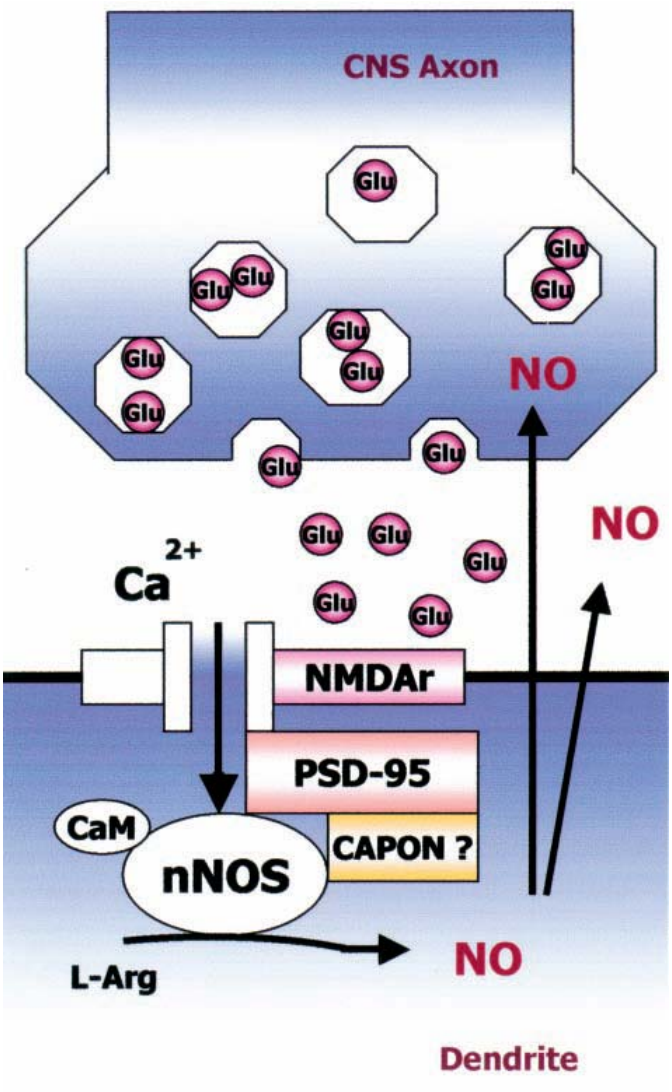


Figure 2.6: NO synthesis (Image 6)

For our purpose is sufficient to know that release of glutamate activates NMDA receptors and the consequence flux of Ca^{2+} entering the ion channel activates nNOS. It is possible that NO bioactivity feeds back to control the presynaptic neuron and the activity of the channel. Just for an entire explanation of the image, PSD-95 is postsynaptic density protein and CAPON is a protein associated with nNOS and regulation of NO formation in neurons. (Esplugues, 2002)

Note: L-Arg (L-Arginine); CaM (calmodulin); Glu (glutamate); nNOS (neuronal NO synthase); NMDAr (NMDA receptor)

The most important regulator of nNOS activity seems to be **free cystolic Ca^{2+}**, which stimulates nNOS through interaction with **calmodulin,** calcium-binding protein. Arrival of action potentials activates voltage-dependent Ca^{2+} channels situated in the neurolemma, and stimulates the release of Ca^{2+} from intracellular stores. This elevates cytosolic Ca^{2+} concentrations above the 400 nM required for calmodulin to bind to nNOS, thereby activating the enzyme. When the concentration of Ca^{2+} falls, it dissociates from the calmodulin, which in turn dissociates from the nNOS, thus activating as a switch that turns the enzyme on and off. This process occurs in the majority of peripheral and in some central nitrergic neurones. However, in the CNS, NO synthesis seems predominantly regulated by the influx of Ca^{2+} through receptor-dependent channels, in particular following postsynaptic stimulation of **NMDA receptors** by the excitatory neurotransmitter, **glutamate** (see Fig. 2.6)**.** (Bredt & Snyder, 1989; Garthwaite et al., 1989)

2.4.4 NITRIC OXIDE IN MITOCHONDRIA

As mentioned above, mitochondria are the important parts of every neuron because they take place in processing of oxygen and production of energy in respiratory chain and Krebs cycle. There is an evidence that NO modulates oxygen consumption in the mitochondria. In particular, nanomolar concentrations of NO inhibit cytochrome oxidase, the terminal hem-containing enzyme in the mitochondrial respiratory chain. Recent evidence demonstrates that this effect is reversible and competitive with oxygen, and suggests that NO is a crucial regulator in the generation of energy and the mediation of cell death by mitochondria. (Beltrán et al., 2000)

2.4.5 NITRIC OXIDE IN CENTRAL NERVOUS SYSTEM

NO is known as a neurotransmitter between nerve cells in CNS. Unlike most other neurotransmitters that only transmit information from a presynaptic to a postsynaptic neuron, the small, uncharged, and fat-soluble nitric oxide molecule can diffuse widely and readily enters cells. Thus, it can act on several nearby neurons, even on those not connected by a synapse.

NO has been proposed as the retrograde messenger which co-ordinates the enhancement of both pre- and post-synaptic mechanism involved in two forms of synaptic plasticity; namely long-term potentiation (see Chapter 2.6.1) and long-term depression (see Chapter 2.6.2). (Esplugues, 2002)

NO has been linked to the release of other neurotransmitters and the effects which they produce, in particular acetylcholine, noradrenaline, dopamine, glutamate, γ-aminobutyric acid (GABA), serotonin, adenosin triphosphate (ATP), carbon monoxide and opioids. (Esplugues, 2002)

It has been suggested that NO modulates gene transcription and translation in neurones and glia. (Esplugues, 2002)

2.4.5.1 SYSTEMIC EFFECT

NO has the complex influences on brain development, memory formation and behavior through regulation of synaptic plasticity.

Inhibition of NO synthesis produces amnesia (Holscher & Rose, 1992), disrupts spatial learning and olfactory memory (Bohme et al., 1993), blunts behavioral performance during task acquisition, and decreases locomotor's activity in habituation tasks (Yamada et al., 1995). NO has also been implicated in neuronal targeting and brain development (Contestabile, 2000), visual processing (Cudeiro & Rivadulla, 1999), discriminative learning (Groll-Knapp et al., 1988), food and drinking behavior (Calapai et al., 1992), thermoregulation (De Luca et al., 1995), opiate tolerance and withdrawal (Mao, 1999), circadian rhythm (Watanabe et al., 1995), sleep (Kapas et al., 1994) and respiratory pattern (Ling et al., 1992).

There is an ever-growing list of peripheral functions in which a role for central NO has been proposed (Esplugues, 2002). There is evidence implicating central NO in the regulation of blood pressure (Togashi et al., 1992), heart rate (Sakuma et al., 1992), stimulated renal sympathetic nerve activity (Sakuma et al., 1992), gastric acid secretion (Esplaques et al., 1996) and motility and motor disruption associated with alcohol abuse (Sandor et al., 1995). Likewise, NO in the CNS appears to be involved in reflexes

leading to a diminished sympathetic output to the periphery and the modulation of various neuroendocrine responses (Esplugues, 2002).

2.4.5.2 ROLE IN PERCEPTION OF PAIN

NO has been implicated at various levels of the nociceptive neural pathways, both peripherally and centrally (Mao, 1999). It is established that synthesis of NO enhances spinal facilitation of the afferent input conveyed to the cortex and subsequently manifested in behavioral responses (Mayer et al., 1999). However the role of NO changes according to the pain stimuli. Inhibition of NO has antinociceptive effects (Kitto et al., 1992) and in contrast, blockade of NO synthesis exacerbates pain in models of mechanical hyperalgesia (Zhuo et al., 1993).

2.4.5.3 ROLE IN NEURONAL DAMAGE AND PROTECTION

The neuronal damage that accompanies cerebral ischemia involves and excessive release of glutamate and a subsequent activation of NMDA receptors that, if maintained for a sufficient period of time, induces a massive influx of Ca^{2+} into the postsynaptic neuron which, in turn, triggers the activation of nNOS and overproduction of NO. In contrast, NO produced by activation of eNOS (Marks et al., 1996) and even NMDA receptors (Fergus & Lee, 1997), plays a protective role in brain ischemia by maintaining regional cerebral blood flow. (Esplugues, 2002)

Inhibition of nNOS with concentrations of NOS inhibitors that do not suppress eNOS activity reduces infarct volume, whereas the use of selective nNOS inhibitors is consistently neuroprotective in models of focal ischemia (Yoshida et al., 1994). Exacerbation of injury seems to occur through inhibition of eNOS with high doses of non-selective NOS inhibitors, which results in deleterious alterations of cerebral blood flow and a subsequent increase in infraction volume. (Esplugues, 2002)

2.4.5.4 ROLE IN DISORDERS OF CNS

There is growing evidence to support a role for NO in the etiology of neurological conditions, including autoimmune and chronic neurodegenerative diseases.

Diseases such as multiple sclerosis and Guillain-Barre syndrome, characterized by widespread loss of myelin, may see their neuronal symptoms exacerbated via the release of NO that accompanies the severe inflammation in the central and peripheral nervous system. (Esplugues, 2002)

In the spinal cord, nNOS expression precedes the death of motor neurones, which follows avulsion of spinal nerve roots (Wu et al., 1994), and pre-treatment with nNOS inhibitors substantially increases the number of surviving neurones (Wu & Li, 1993).

The excessive release of both glutamate and NO, coupled with mitochondrial dysfunction and oxidative stress, has been implicated in a number of neurodegenerative diseases. This highlights a potential therapeutic role for specific NOS inhibitors in their pharmacological control (Hobbs et al., 1999).

For instance, nNOS is induced in various cortical regions following epileptic seizures (Huh et al., 2000). NOS neurones are spared in Alzheimer's disease and NOS inhibitors provide neuroprotection against toxicity in primary cortical cultures (Hyman et al., 2000). Inhibition of nNOS markedly reduces the loss of dopamine neurones and clinical symptoms in a baboon model of Parkinson's disease (Hantraye et al., 1996) and inhibition of NOS is protective in models of Huntington's disease (Deckel, 2001). Significant changes in nNOS activity in the cerebrum and cerebellum follow the administration of metals such as aluminum and mercury, and suggest an involvement of this mediator in cerebral diseases induced by metals (Cucarella et al., 1998).

There are increased levels of NO production in viral and bacterial infections such as meningitis, and a role for NO has been clearly indicated in the disruption of the blood-brain barrier during inflammatory conditions (Brian et al., 1995).

NO has been implicated as a potential mediator of microglia-dependent primary demyelization, a hallmark of multiple sclerosis, and iNOS induction has been noted in the brains of patients with this autoimmune disease. NO may also be involved in the pathogenesis of sporadic amyotrophic lateral sclerosis and that of AIDS dementia (Bo et al., 1994).

2.4.6 NO IN THE PERIPHERAL NERVOUS SYSTEM

NO has a leading role as an inhibitory neurotransmitter of peripheral non-adrenergic, non-cholinergic nerves. Peripheral nitrergic nerves have a widespread distribution, and are particularly important in that they produce relaxation of smooth muscle in the gastrointestinal, respiratory, vascular and urogenital systems. It is generally assumed that free NO is the transmitter substance released by nitrergic nerves. (Esplugues, 2002)

2.5 IMAGING TECHNIQUES OF NITRIC OXIDE

2.5.1 CHEMILUMINESCENT REACTION INVOLVING OZONE

The concentration of NO can be determined using a simple chemiluminescent reaction involving ozone. A sample containing nitric oxide is mixed with a large quantity of ozone. The nitric oxide reacts with the ozone to produce oxygen and nitrogen dioxide. This reaction also produces light (chemiluminescence), which can be measured with a photodetector.

$$NO + O_3 \rightarrow NO_2 + O_2 + \text{light}$$

The amount of light produced is proportional to the amount of nitric oxide in the sample. (Fontijn et al., 1970)

2.5.2 FLUORESCENT DYE

A group of fluorescent dye indicators which are also available in acetylated form for intracellular measurements exist. The most common compound is **4,5-diaminofluorescein (DAF-2)**. (Nagano & Yoshimura, 2002)

Another one is represented by **diaminoanthraquinone (DAA)**. A method for the direct measurement of intracellular NO production stimulated by penicillin G in cultured hippocampal neurons with this dye using laser scanning confocal microscopy was developed. Intracellular DAA fluorescence could specifically represent NO production based on two facts: (1) 3-morpholinosydnonimine, a NO donor, could dose-dependently increase DAA fluorescence; and (2) haemoglobin, a NO scavenger, could inhibit the increase of DAA fluorescence. These results revealed that DAA could be used to indicate real-time and kinetic intracellular NO production of hippocampal neurons with higher sensitivity, specificity and accuracy. (Chen & Sheng & Zheng, 2001)

DAQ (1,2-diaminoanthraquinone) is the aromatic vicinal and has been already used to determine changes in NO production by imaging techniques. The dark violet DAQ possesses no direct fluorescence. NO reacts with the aromatic amino groups of DAQ at neutral pH and in the presence of oxygen. The final reaction product (ATD) shows fluorescence. (Schuchmann et al., 2002)

2.6 NEUROPLASTICITY

Neuronal activity can modify the behavior of neural circuits by one of three mechanisms:

- by modifying the strength or efficacy of synaptic transmission at preexisting synapses,
- by eliciting the growth of new synaptic connections or the pruning away of existing ones,
- by modulating the excitability properties of individual neurons. (Malenka, 2002)

Neuroplasticity is the process of changing of neurons, the organization of their networks, and their function via new experiences. This idea was first proposed in 1890 by William James. The evidence for neurogenesis is mainly restricted to the **hippocampus** and **olfactory bulb**, but current research has revealed that other parts of the brain, including the cerebellum, may be involved as well. (Wall & Xu & Wang, 2002)

In the rest of the brain, neurons can die, but they cannot be created. However, there is now ample evidence for the active, experience-dependent reorganization of the synaptic networks of the brain involving multiple interrelated structures including the cerebral cortex. The specific details of how this process occurs at the molecular and ultrastructural levels are topics of active neuroscience research.

Neuroplasticity is an essential issue that supports the scientific basis for treatment of brain injury with goal-directed experiential therapeutic programs in the context of rehabilitation approaches to the functional results of the injury.

The subunit of neuroplasticity is the term **synaptic plasticity** which is the ability of the connection, or synapse, between two neurons to change in strength. It refers to the first of the mechanisms mentioned in the beginning of chapter. It is one of the important neurochemical foundations of learning and memory.

2.6.1 LONG - TERM POTENTIATION (LTP)

LTP is a long-lasting enhancement in signal transmission between two neurons that results from stimulating them synchronously (Cooke & Bliss, 2006). It is one of phenomena underlying the synaptic plasticity and it represents the ability of synapses to improve their connectivity in their strength. (Paradiso et al., 2007).

LTP is thought to be a synaptic correlate of learning and memory, and is most pronounced in higher brain centers involved in cognitive functions, particularly in the cerebral cortex and hippocampus. (Esplugues, 2002)

The forms of LTP differ in mechanisms depending on the regions of the brain when it occurs. For example in hippocampus, some of LTP types depend on NMDA receptors and others may depend on metabotropic glutamate receptors. NMDA receptor-dependent LTP exhibits specific properties including input specificity, associativity, cooperativity and persistence. The whole process in commonly divided into three phases: short-term potentiation, early LTP and late LTP. There is little known about the mechanisms of the first one but early LTP occurs when the concentration of Ca^{2+} inside the postsynaptic cell exceeds a critical threshold which can be evoked experimentally by applying a few trains of high-frequency stimuli; all processes realize through NMDA receptors (that is why called NMDA receptor-depend LTP) when activated by sufficient depolarization and bound by glutamate. Late LTP is induced by changes in gene expression and protein synthesis.

2.6.2 LONG - TERM DEPRESSION (LTD)

LTD is an activity-dependent reduction in the efficacy of neuronal synapses lasting hours or longer (Massey & Bashir, 2007). It occurs in many areas of the CNS with varying mechanisms depending upon brain region and developmental progress. Cerebellum and hippocampus belong between the best characterized regions in this phenomenon. LTD is one of several processes that serve to selectively weaken specific synapses in order to make constructive use of synaptic strengthening caused by LTP. This is necessary because, if allowed to continue increasing in strength, synapses would

ultimately reach a ceiling level of efficiency, which would inhibit the encoding of new information (Purves et al., 2008).

Physiological principle of LTD in hippocampus is based on controlling of influx of postsynaptic Ca^{2+} through NMDA receptors because the process of depression depends on the timing and frequency of Ca^{2+} influx and it occurs only in small and slow rises of postsynaptic Ca^{2+} levels between Schaffer collaterals and CA1 pyramidal cells. Experimentally, it is elicited by repetitive stimulation for extended time periods at a low frequency.

2.7 EPILEPSY

2.7.1 DEFINITION

Epilepsy is a common chronic neurological disorder characterized by recurrent unprovoked seizures. These seizures are transient signs and/or symptoms of abnormal, excessive or synchronous neuronal activity in the brain.

About 50 million people worldwide have epilepsy, with almost 90% of these people being in developing countries. Epilepsy is more likely to occur in young children or people over the age of 65 years; however it can occur at any time.

Not all epilepsy syndromes are life long - some forms are confined to particular stages of childhood. Epilepsy should not be understood as a single disorder, but rather as syndromic with vastly divergent symptoms but all involving episodic abnormal electrical activity in the brain. (Fischer et al., 2005)

2.7.2 PATHOPHYSIOLOGY

2.7.2.1 PATHOPHYSIOLOGY IN POPULATION

Changes that occur during epileptogenesis are poorly understood but are thought to include cell death, axonal sprouting, reorganization of neural networks, alterations in the release of neurotransmitters, neurogenesis. These changes cause to the neurons to become hyperexcitable and can lead to spontaneous seizures. (Herman, 2006)

Brain regions that are highly sensitive to insults and can cause epileptogenesis including temporal lobe structures such as the hippocampus, the amygdala and the piriform cortex. (Aroniadou-Anderjaska, 2008)

Gene mutations

Gene mutations have been linked to some types of epilepsy. Several genes that code for protein subunits of voltage-gated and ligand-gated ion channels have been

associated with forms of generalized epilepsy and infantile seizure syndromes. Several ligand-gated ion channels have been linked to some types of frontal and generalized epilepsies. One speculated mechanism for some forms of inherited epilepsy are mutations of the genes which code for sodium channel proteins; these defective sodium channels stay open for too long thus making the neuron hyper-excitable. Glutamate, an excitatory neurotransmitter, may thereby be released from these neurons in large amounts, which by binding with nearby glutamanergic neurons, triggers excessive calcium (Ca^{2+}) release in these post-synaptic cells. Such excessive calcium release can be neurotoxic to the affected cell. The hippocampus, which contains a large volume of just such glutamanergic neurons (and NMDA receptors, which are permeable to Ca^{2+} entry after binding of both sodium and glutamate), is especially vulnerable to epileptic seizure, subsequent spread of excitation, and possible neuronal death. Another possible mechanism involves mutations leading to ineffective GABA (the brain's most common inhibitory neurotransmitter) action that results break-down of inhibitory mechanisms in CNS and induces uncontrolled excitation in neurons. Epilepsy-related mutations in some non-ion channel genes have also been identified. (Hopkins et al., 1995)

Chemical agents

Imbalance of intrinsic chemical compounds, natural or artificial, can activate cascade of processes that can finally lead to seizure activity. These substances are called neurotoxins and their exposure occurs for example in chemotherapy, radiation treatment, drug therapies, but as well as in exposure of heavy metals, certain food, industrial or cleaning solvents, cosmetics, etc. Repeated exposures to some pesticides have been shown to induce seizures in both humans and animals. One mechanism, proposed for this, is called excitotoxicity. (Hopkins et al., 1995)

Traumatic brain injuries

Seizures may also occur after traumatic brain injury; these are known as post-traumatic seizures (PTS). In the period between a brain injury and onset of epilepsy, brain cells may form new synapses and axons, undergo apoptosis or necrosis, and experience altered gene expression. In addition, damage to particularly vulnerable areas of the cortex such as the hippocampus may give rise to post-traumatic epilepsy (PTE). In other cause, blood that gathers in the brain after an injury may damage brain tissue

and thereby cause epilepsy; or the products that result from the breakdown of hemoglobin from blood may be toxic to brain tissue. The “iron hypothesis” holds that PTE is due to damage by oxygen free radicals.

Abnormalities in the release of neurotransmitters may play a role in the development of PTE. Traumatic brain injury can lead to the excessive release of glutamate and other excitatory neurotransmitters and it leads to excitotoxicity, damage to brain cells through overactivation of the biochemical receptors that bind and respond to excitatory neurotransmitters. This overactivation of glutamate receptors damages neurons.

Important independent risk factors for PTE include acute intracerebral hematoma (especially subdural hematoma), brain contusion, increased injury severity (as reflected by loss of consciousness or posttraumatic amnesia lasting more than 24 h), occurrence of early posttraumatic seizures, and being older than 65 years at the time of injury. (D`Ambrosio & Perucca, 2004)

2.7.2.2 PATHOPHYSIOLOGY IN EXPERIMENTAL ANIMAL MODELS

Because of ethical codex, the scientists cannot measure all recordings from neurons of living human brain. But properly controlled experiments can be successfully done with animal models of epilepsies.

In vivo

In vivo (Latin for “within the living”) is experimentation using a whole, living organism. *In vivo* imaging provides a noninvasive method for imaging biological processes in live animals in order to understand metabolic processes, effects of drugs or disease progression. Animal models can be studied either acutely (in which the animal is sacrificed at the end of experiment) or chronically (where the same animal can be studied repeatedly over long periods). It is useful mostly for long-term experiments in one animal and long-term collection of data in the same conditions because it is still the same organism which progresses in time.

Models of epilepsy can be induced by application of **convulsant substances** to brain of animals. All these convulsant substances are acting through ionic channels or transmitters or metals. Metals such as aluminum, iron or cobalt produce these focal models (Hopkins et al., 1995); or the neurotoxin kainic acid appears to have a preferential excitable effect on hippocampus when injected systematically (Nadler, 1981). Other options are in using the chemicals such as Bicuculline (see Chapter 2.7.4.7), Carbachol (see Chapter 2.7.4.8), 4-aminopyridine (see Chapter 2.4.7.9), etc. in the same way like *in vitro* studies. In general, we can say that models used *in vitro* are suitable for *in vivo* studies too and in additional, *in vitro* experience should prepare good conditions and adequate results in measurement for following *in vivo* studies.

Or the seizure activity can be elicited by electrode stimulation. The kindling model represents this option in research. It is a widely used model for the development of seizures and epilepsy. The word **kindling** is a metaphor: the increase in response to small stimuli is similar to the way small burning twigs can produce a large fire. It means that duration and behavioral involvement of induced seizures increases because seizures are induced repeatedly. In this model a stimulating electrode is put in excitable brain tissue, most commonly the amygdala, and brief shocks are given on a daily basis. After few days or weeks it progresses to full epileptiform discharges and behavioral consequences (Purpura et al., 1978).

In vitro

Physiological experiments are difficult in living whole brain tissue in terms of low accessibility, lack of control of the neuronal micro-environment, confounding effects of anesthetics and mechanical instability from cardiorespiratory pulsations. Experiments performed in vitro minimize these difficulties at the cost of isolation of brain tissue from its usual communicating structures.

In vitro (Latin for “within the glass”) experiment is performed not in a living organism but in a controlled environment. *In vitro* electrophysiological investigation can be carried out on normal tissue that is made epileptic “in the dish” or on tissue removed from animal models of chronic epilepsy. Preparations can be acute (slices, dissociated cells, etc.) or long-term (e.g. tissue cultures (Pitkänen et al., 2006). The most

useful of the *in vitro* techniques for the study of epilepsy has been the explanted hippocampal slice (Hopkins et al., 1995).

Transverse slicing of the hippocampus preserves its laminar circuitry which is very useful for these experiments. Addition of chemicals to the media allows control of the microenvironment, and intracellular recordings are relatively easy to obtain. We can evoke bursting which is taken as a model of epileptiform activity by electrode stimulation, addition of pharmaceuticals or changing intracellular and extracellular conditions of neurons (for more details see 2.7.3.2, 2.7.4 or Methods).

2.7.3 ACUTE SLICES

Animal models of epilepsy are mostly used to investigate fundamental neuronal mechanisms of both abnormal (usually epilepsy related) and normal brain function. Animal models are also important, however, for research designed specifically to devise new diagnostic approaches or to test the efficacy of new antiepileptic drugs or other novel therapeutic interventions. Animal models are essential for understanding basic mechanisms of epilepsy (structural and functional abnormalities), investigation of pharmacotherapy and alternative therapies and developing a prevention of epilepsy, antiepileptogenesis, because none of current antiepileptic drug is known to be antiepileptogenic.

The most popular *in vitro* preparations are acute brain slices, consisting of 200- to 600-μm thin sections of living brain tissue, which can be obtained from any species with a complex brain.

2.7.3.1 POTENTIAL PROBLEMS

Some compoundable problems must be taken into account by investigators using slice preparations:

- Inevitably such preparations have undergone a period of ischemia. Further, many fiber projections entering and leaving the brain slice are severed.

- Moreover the thickness of the preparation requires a nutrition supply that is dependent on exchange by diffusion from the artificial cerebrospinal fluid (ACSF) and diffusion processes are much less efficient than in real conditions.
- As a result of the acute lesion, and in analogy to *in vitro* observations after acute transaction of the spinal cord, there is a drastic reduction of spontaneous synaptic activity.

The last point is less important where physiological synaptic activity can be maintained by intraslice circuitry (e.g. in hippocampal formation) and sufficient oxygen and glucose supply is well-balanced by saturation with 95% oxygen and high glucose content. (Pitkänen et al., 2006)

2.7.3.2 PREPARATION, MAINTENANCE AND RECORDING

Most laboratories used a tissue chopper for cutting the tissue into thin slices but later, vibratomes were adopted in many laboratories because they appear to make slices with less mechanical damage. The slices are maintained in the static bath of gassed ACSF, placed on the lens paper, which facilitates transfer to the recording chamber. Another important issue of experiment is the temperature at which is the tissue maintained because SLE is difficult to induce at temperatures below 33°C. And another one is the composition of the ACSF (see Methods for more details). The osmolarity of the solution should be about 290mOsm and Ca^{2+}, Mg^{2+} and K^{+} concentrations must be carefully adjusted because even small variations in concentration of these ions have marked effects on neuronal activity. (Pitkänen et al., 2006)

Slice recordings can be performed in an **interface chamber** or in a **submerged chamber**. First, an interface chamber is characterized by presentation of two environments in the chamber when the slice placed on the bearing plate overflows by ACSF on a lower side and on the upper is surrounded by atmosphere of humidified gas. Second, a submerged chamber isolates a slice from atmosphere conditions by submerging in ACSF and the solution overflows the slice in both sides, above and below. Maintaining in submerged chamber has proven much harder because there is a possibility of problem with diffusion of oxygen and interference sounds in recording developed by flow of ACSF. But, if successful, would offer important experimental

advantages over interface conditions, including faster exchange of pharmacological agents, visually guided patch-clamp recordings (recordings used for measurement of membrane current at single ion channel or multiple channel at once) and advanced imaging techniques. (Hájos et al., 2009)

Submerged chamber design is modified in two ways. First, in order to reduce the volume of the chamber and direct the superfusion fluid over slice, an inert plastic insert is used. The second modification allows a double superfusion system to be used. In this design, the slice is placed on a mesh glued between two plastic rings. Two separate fluid inlets allow ACSF to flow separately above and below the slice. (Hájos et al., 2009)

In the recording chamber, we have different possibilities to use pharmacological agents to influence function of the nervous tissue. We can incubate the slice in the recovery chamber or add substances into the perfusion during recording.

As mentioned above, the most useful model *in vitro* for investigation in epilepsy research seems to be a hippocampal transverse slice of rat because of its unidirectional connectivity and similarity to the human structure and function.

2.7.4 EXPERIMENTAL MODELS

The mechanisms, which induce an epileptiform activity on hippocampal slices *in vitro* experiments, make use of ionic changes in extracellular conditions (by lowering or increasing the concentration of specific ions in ACSF), addition of pharmaceuticals into the ACSF, stimulating the slice by microelectrode, or combination of all mentioned methods. The success in inducing an activity depends mostly on accurate concentration of ACSF and particularly on setup of each laboratory.

2.7.4.1 GABAergic DISINHIBITION

In the absence of GABAergic neurotransmission, synchronized inter-ictal-like (inter-ictal means period of time between seizures) burst can occur. These bursts depend on fast glutamatergic neurotransmission to activate AMPA and NMDA receptors. In general, the activation of ictal-like (ictal means a period of time during an actual

seizure) events *in vitro* requires additional pharmacologic manipulation, such as elevation K^+ (see Chapter 2.7.4.6) or addition of Bicuculline (see Chapter 2.7.4.7), competitive antagonist of $GABA_A$ receptors (see Chapter 2.3.3). (Pitkänen et al., 2006)

2.7.4.2 KAINIC ACID

Kainic acid (KA) is a specific agonist for the kainate receptor used as an ionotropic glutamate receptor which mimics the effect of glutamate and has presynaptic and postsynaptic effects. KA can directly depolarize neurons that express KA receptors, such as CA3 pyramidal cells and interneurons, leading them to generate action potentials. Despite its epileptogenicity *in vivo*, it has not been extensively used to study the intention and propagation of seizure-like discharges in the hippocampal slice preparation. (Pitkänen et al., 2006)

2.7.4.3 BLOCK OF K^+ CHANNELS

Blocking K^+ channels with 4-Aminopyridine (see Chapter 2.7.4.9), an organic compound, its molecule is one of the three isomeric amines of pyridine; it is a relatively selective blocker of voltage-activated K^+ channels; it prolongs action potentials) induces interictal-like and ictal-like discharges in hippocampus. (Pitkänen et al., 2006)

2.7.4.4 LOW-Ca^{2+} MODEL

Lowering Ca^{2+} abolishes neurotransmission and results in spontaneous paroxysmal discharges. These events arise focally and spread through CA1 region. This model may be particularly valuable for the study of synchronization mechanisms and for investigating interventions to abort or stop these discharges. (Pitkänen et al., 2006)

In our research, there is a tendency for investigation of five models of epileptiform activities to demonstrate a role of NO, respectively to demonstrate and induce SLEs in vitro in our setup in submerged chamber which is required for imaging techniques and NO detection…..low-Mg^{2+} (magnesium), high-K^+ (potassium), Bicuculline, Carbachol and 4-Aminopyridine model. Performance of the models was

done on hippocampal slices (see Methods for more details). There is a list of experiments made in each model for purpose of confrontation with previous researches.

2.7.4.5 LOW-Mg^{2+} MODEL

Low-Mg^{2+}-induced epileptiform activity results from increased synaptic excitation due to facilitated activation of NMDA receptors (Mody et al., 1987). NO decisively modulates low-Mg^{2+}- induced epileptiform activity. The induction of low-Mg^{2+} epileptiform activity is associated with an increase in NO production. The maintenance of low-Mg^{2+}-induced epileptiform activity requires an increased level of NO concentration: reduction of NO level can block activity, whereas elevation of NO levels can induce activity. (Schuchmann, 2002)

The studies show increasing NO production following removal of Mg^{2+} ions from the perfusion medium before the first seizure-like events (SLEs) arise. Reducing the endogenous NO production using the NOS inhibitor L-NAME suppressed SLEs and therefore underlines the meaning of NO for low-Mg^{2+}-induced epileptiform activity. Furthermore, suppressed epileptiform activity due to blocked endogenous NO production was reactivated by exogenously applied NO. Finally, the required endogenous or exogenous amounts of NO for the induction of low- Mg^{2+}-induced epileptiform activity were found to be approximately equal. (Schuchmann, 2002)

It is suggested that the NMDA/NOS system is of primary importance in a variety of physiological and pathological processes of the brain (Contestabile, 2000). Lowering the extracellular Mg^{2+} concentration has been shown to induce spontaneous epileptiform activity by removal of the voltage-dependent Mg^{2+} block from the NMDA receptor (Mody et al., 1987).

Enhanced NO production during low-Mg^{2+}- induced epileptiform activity may influence the decrease in neuronal energy supply and may accelerate the transition into the resistant late activity. The finding of an increased frequency of SLEs after elevation of NO concentration corresponds well with hypotheses that attribute the transition to late activity in the low-Mg^{2+} model to an energetic failure (Schuchmann, 2002). Furthermore, mitochondria have been demonstrated to possess an autonomous system to generate NO, localized in the inner mitochondrial membrane, which may take part in

the control of ATP production (Giulivi et al., 1998). There is a suspect of functional role for mitochondria in epileptiform activity via neuronal energy metabolism, which may be modulated by this mitochondrial NO synthesis. (Schuchmann, 2002)

Schuchmann et al., 2002, measured the production of NO during low-Mg^{2+}-induced epileptiform activity in rat hippocampal-entorhinal cortex slices in submerged chamber. They induced spontaneous SLEs which developed during prolonged washout of Mg^{2+} ions from ACSF (zero Mg^{2+} model). In 7 of 7 brain slices spontaneous series of SLEs was recorded. The frequency of low-Mg^{2+}-induced SLEs was 0.53 ± 0.19 min^{-1} and their duration was 22 ± 1.8 s.

Low-Mg^{2+} model results a long-term activation of NMDA receptors so that is far away from reality of epileptic activity in vivo, that is why we decided to not use this model for following researches.

2.7.4.6 HIGH-K^+ MODEL

Because K^+ currents play an essential role in controlling neuronal excitability, it was initially hypothesized that elevations of extracellular K^+ are the cause of epileptiform activity. According to the potassium accumulation hypothesis, extracellular K^+ fluctuates around a stable baseline during physiological activity levels. A transient increase triggers the occurrence of a seizure during which K^+ further accumulates. As a result, neurons become even more depolarized, fire more APs, and release even more K^+ into the extracellular space. Eventually, these run-away dynamics (positive feedback) come to an end when the neurons are so depolarized that they can no longer spike due to sodium channel inactivation. At this point, the seizure terminates. (Fröhlich et al., 2008)

In addition, this model relates with the Leão`s phenomenon call spreading depression (SD) which can be set off by high K^+ levels in extracellular space. This phenomenon is characterized by rapid and nearly complete depolarization of a sizable population of brain cells with massive redistribution of ions between intracellular and extracellular compartments and propagates slowly as a wave in brain tissue. SD can be produced by positive feedback achieved by increase of extracellular K^+ concentration that activates persistent inward currents which then activate K^+ channels and release

more K^+. (Somjen, 2001) These levels of extracellular K^+ are so high (up to 100mM!) that they lead into the depression of neuronal activity; normally it is about 3 mM and during evoked SLE there is concentration up to 10mM of K^+.

Increasing in $[K^+]_0$ also directly affect synaptic inhibition. The equilibrium potential of $GABA_A$ receptors shifts toward less negative values in elevated $[K^+]_o$. (Fröhlich et al., 2008)

This model is particularly useful because it causes an elevation of the general excitability of all neuronal networks (Pitkänen et al., 2006).

Fröhlich et al., 2008, described in their work that elevation of K^+ concentration in the ACSF to 7.5 or 8.5mM was sufficient to trigger both periodic network activation ("interictal spikes") and in some cases events resembling electrographic seizures with a "tonic" firing and "clonic" bursting phase in the hippocampus. Furthermore, SLEs occurred depending on the degree of $[K^+]_o$ increase evoked by extracellular electrical stimulation or focal potassium injection in a high- K^+ and low-Ca^{2+} model. Together, these ionic models illustrate that elevated $[K^+]_o$ is clearly sufficient to trigger synchronized oscillatory activity at various frequencies in the hippocampal networks *in vitro*.

Leschinger et al., 1993, studied some of the physiological and pharmacological properties of an in vitro model of epileptic seizures induced by elevation of $[K^+]_o$ (to 8mM and 10mM) in combination with lowering of $[Mg^{2+}]_o$ (to 1.4mM and 1.6mM) and $[Ca^{2+}]_o$ (to 0.7mM and 1mM) in rat hippocampal slices. The resulting data suggest that the high-K^+ model of epileptiform activity is a good model of focal convulsant activity.

Sagratella et al., 1987, made a result in their research that increase of the potassium concentration up to 8mM in the superfused solution of rat hippocampal slices leads to the development of an epileptiform bursting. They made several other measurements with antagonist L-PIA and its ability to block the potassium induced epileptiform activity.

2.7.4.7 BICUCULLINE

Bicuculline is a light-sensitive competitive antagonist of $GABA_A$ receptors. Since it blocks the inhibitory action of GABA receptors, the action of Bicuculline mimics epilepsy.

This property is utilised in laboratories across the world in the *in vitro* study of epilepsy, generally in hippocampal or cortical neurons in prepared brain slices from rodents. This compound is also routinely used to isolate glutamatergic (excitatory amino acid) receptor function.

The action of Bicuculline is primarily on the ionotropic $GABA_A$ receptors, which are ligand-gated ion channels concerned chiefly with the passing of chloride ion across the cell membrane, thus promoting an inhibitory influence on the target neuron. These receptors are the major targets for benzodiazepines and related anxiolytic drugs.

In addition to being a potent $GABA_A$ receptor antagonist, bicuculline can be used to block Ca^{2+}-activated potassium channels. (Khawaled et al., 1999)

More recently, blockage of K^+ channels and prolongation of Ca^{2+} action potentials has been also suggested as a possible mechanism of seizure induction by Bicuculline (Engel & Pedley, 2008).

Desalvo et al., 1995, studied BOLD fMRI changes in aneshetized Wistar rats during bicuculline-induced tonic-clonic seizures. Bicuculine was injected systematically and seizure activity was observed on EEG. In the work of Wu et al., 1994, Bicuculline led to a marked increase in the spontaneous discharged rates of 6/7 (85.71%) neurons in an epileptiform pattern.

2.7.4.8 CARBACHOL

Carbachol is a drug that binds and activates the acetylcholine receptor. Thus it is classified as a cholinergic agonist. Furthermore, Carbachol is a parasympathomimetic (mimicking the parasympathetic nervous system) that stimulates both muscarinic and nicotinic receptors and mimics epilepsy in this process.

Cruickshank et al., 1994, made several experiments to determine the types of acetylcholine receptors involved in the initiation of epileptic seizures from the zona incerta, in subthalamus, and surrounding structures by cholinergic stimulation in rats. Unilateral intracerebral microinjection of the mixed muscarinic and nicotinic agonist carbachol produced generalized seizures in 12 of 20 rats studied. (Cruickshank et al., 1994). The same result achieved Peterson and Armstrong, 1999, in their intention to determine the role of cholinergic mechanism by the microinjection of Carbachol into the nucleus reticularis pontis oralis. Bawin et al., 1994, investigated the role of nitric oxide in controlling the rate of occurrence of muscarinic rhythmic slow activity episodes induced by Carbachol in rat hippocampal slices.

2.7.4.9 4-AMINOPYRIDINE

4-Aminopyridine (4-AP) is an organic compound and its molecule is one of the three isomeric amines of pyridine. The largest scale industrial application of 4-aminopyridine is as a precursor to the drug Pinacidil, which affects potassium ion channels. It is an extremely poisonous bird poison and it is highly poisonous to mammals too.

4-Aminopyridine (4-AP) is a voltage-gated, fast potassium channel blocker capable of improving axonal conduction by facilitating the propagation of APs in demyelinated nerve fibers. It does not replace damaged myelin but users of 4-AP report dramatic improvement in a number of symptoms especially paraesthesia. It has been used clinically in Lambert-Eaton syndrome and multiple sclerosis (MS). It has been shown to improve visual function and motor skills and relieve fatigue in patients with MS.

It is used primarily as a research tool, in the laboratory; 4-AP is a useful pharmacological tool in studying various potassium conductances in physiology and biophysics. It is a relatively selective blocker of members of voltage-gated K^+ channels. Some reports have shown that overdoses with 4-AP can lead to paraesthesia, seizures, and atrial fibrillation. For the purpose of our study is the most essential that it cases seizures.

Schuchmann et al., 1999, induced an epileptiform activity in brain slices of Wistar rats and made it in two experimental models, low-Mg^{2+} and addition 250μM 4-AP, for the purpose of their experiment in relative energy failure in entorhinal cortex during these SLEs. After exposure to 250μM 4-AP, SLEs were observed at a frequency of 0,32 ± 0,1min^{-1}; the duration was 19 ± 1.5 s. Late recurrent discharges were not observed in 9 out of 9 slices after a maximal exposure time of >3h.

2.7.5 ELECTROPHYSIOLOGY

In a seizure focus, every single neuron has the paroxysmal depolarization shift (PDS), which is a stereotypic and synchronized electrical response. Activation of excitatory glutamate-mediated channels such as AMPA and NMDA receptor channels as well as voltage-gated Ca^{2+} primarily cause the depolarizing phase. The PDS consists of a sudden, large 20 - 40mV, long-lasting (50 - 200ms) depolarization, which triggers a train of action potentials at the peak of the PDS. The afterhyperpolarization appears after PDS.

Voltage-gated K^+, Na^+, Ca^{2+} channels and the others intrinsic membrane properties of the neuron as well as synaptic inputs from excitatory (glutamatergic) and inhibitory (GABAergic) neurons respectively shape the PDS and the afterhyperpolarization.

A typical cortical pyramidal neuron responds to excitatory input in an excitatory postsynaptic potential (EPSP) followed by an inhibitory postsynaptic potential (IPSP). The PDS can be thus viewed as a gross exaggeration of the normal depolarizing and hyperpolarizing components observed in neurons in a typical cortical circuit.

Hippocampal neurons in the CA3 region exhibit PDS behavior under normal conditions and that is the way how they differ from most neurons. PDS is ended by the afterhyperpolarization. The generators of the afterhyperpolarization are primarily calcium- and voltage-dependent K^+ channels as well as GABA-mediated chloride and K^+ conductance. The Ca^{2+} entry through voltage-dependent Ca^{2+} channels and NMDA channels triggers calcium-dependent, particularly voltage-dependent Ca^{2+}. The synaptic activity drives the sequences of depolarization and hyperpolarization that is why many

convulsants act by either enhancing excitatory or blocking inhibition which can be used in pharmacological treatment of epilepsy. The effective anticonvulsants are drugs that block excitation of enhance inhibition.

As a result of exitotoxic lesion of hippocampal sclerosis is the most common pathologic finding in TLE. Hippocampal sclerosis involves hippocampal cell loss in the CA1 and CA3 regions and the dentate hillus.

The break down of surround inhibition is the mechanism of seizure, but we still don't know what causes a seizure to occur at any particular moment. There are many clinical factors such as stress and sleep derivation triggering seizures to in some patients, thus it is possible that diffuse cortical cholinergic, noradrenergic or serotonergic projections may play a crucial modulatory roles in some cases. Sensory stimuli such as flashing lights can also trigger seizures and we suggest that repeated excitation of circuits a frequency dependent manner providing another possible cellular mechanism for changing network excitability. (Konopková, 2004)

2.7.6 THE ROLE OF NITRIC OXIDE

The functional involvement of NO in epileptiform activity has been demonstrated by several researchers. However, the role of NO in the expression of epileptiform activity is still unclear. NO has been proposed as an endogenous proconsulvant (Yasuda et al., 2001) as well as an anticonsulvant (Ferraro et al., 1997).

In the present studies, there is a tendency to investigate the role of NMDA/NOS system on epileptiform activity in the entorhinal cortex. The functional role of NO in epilepsy is controversial (Schuchmann, 2002). On the one hand, NO is known to activate guanylate cyclase, which increases cGMP levels and finally may cause an elevation in presynaptic release of glutamate (Garthwaite, 1991). Furthermore, increase in NO by the application of the NOS activator L-arginine has been shown to potentiate NMDA-induced seizure activity in the prepiriform cortex (De Sarro et al., 1993). On the other hand, NO has been suggested to cause a negative feedback on NMDA receptors by alteration of the receptor redox modulatory site (Lipton et al., 1993) and via blockage of the NMDA recognition site of the receptor (Manzoni et al., 1992).

Several studies have suggested that some of the physiological and pathophysiological NO effects reveal a NO-modifying action on mitochondrial function. NO has been demonstrated to disturb intracellular Ca^{2+} homeostasis and mitochondria functions (Doutheil et al., 2000). Moreover, it has been demonstrated that NO causes a Ca^{2+}/calmodulin-dependent synaptic potentiation in hippocampal CA1 neurons, which could be blocked by L-NAME, NOS inhibitor (Ko & Kelly, 1999). In addition, there may be a synergic role for intracellular Ca^{2+} and NO in causing mitochondrial dysfunction (Keelan et al., 1999). Furthermore, increased NO concentration has been shown to inhibit mitochondrial functions and neuronal energy metabolism (Brorson et al., 1999).

2.8 EPILEPSY AND REHABILITATION

Epilepsy should not be understood as a single disorder, but rather as syndromic with vastly divergent symptoms but all involving episodic abnormal electrical activity in the brain. Rehabilitation in epilepsy includes all aspects of daily living such as medical treatment; social care, mainly a person's ability to cope in society; psychological intervention like a depression and stress; and neuropsychological aspects like memory difficulties or cognitive problems.

Using of physical rehabilitation in epilepsy is less frequent than in other neurological disorders. Or have you ever heard about physical rehabilitation in epilepsy? There is a little mention about this in literature, rather less than more. But the epilepsy is a neurological disorder like another one so it deserves little more attention of physiotherapy. A minority of individuals, however, may have physical disabilities, the most common being hemiparesis and ataxia, for which assessment and intervention by a physiotherapist form an important part of rehabilitation process. Moreover, physiotherapy can be taken in account in process of prevention of frequency in epileptiform seizures. Physical activities of the patients with epilepsy play an important role in releasing of stress and depression and support a solid healthy life style as the most important part of prevention. In addition, physiotherapy should prevent the side effects of drugs using in epilepsy (antiepileptics; anticonvulsants) such as putting on weight, ataxia, vertigo, headache, nystagmus, paraestheazia, problems in cognitive functions (dysarthria), etc. Then the therapeutical procedures should be aimed on weight reduction by therapeutical physical training; and supervision of movement which can be achieved by sensomotoric stimulation, proprioceptive neuromuscular stimulation, balance training and other methods used in daily routine. Problems in cognitive function can be dissolved for example by stimulation or soft massages in oral facial region or Sister Kenny Method, all with cooperation of speech therapist.

Moreover, epilepsy is the most common chronic disorder of the CNS and we know that it performs as a symptom of many other neurological disorders. For example cerebral palsy, in numbers, seizures occur in about half of those with this disorder and are much more common in tetraplegic (94%) and hemiplegic cerebral palsy (34%) than

in extrapyramidal form, although epilepsy is also common in the mixed forms. Epilepsy is related to structural damage, for example in congenital hemiparesis it occurs in half of those with cortical/subcortical atrophy, less commonly in those with unilateral ventricular dilatation. Epilepsy also occurs in about a quarter of those with meningomyelocele and hydrocephalus and there is also evidence that after head injury or intracranial surgery some patients can be identified to have a substantial risk of developing epilepsy. (Hopkins et al., 1995)

So, as you can see, it is a large amount of patients with specific diagnosis whom the physiotherapist meets in his routine, both in the hospital and private practice and at least, he should be prepared to afford them an adequate first aid. All of these arguments make the physiotherapy in epilepsy an essential part of global approach of rehabilitation.

3. AIMS AND HYPOTHESIS

3.1 AIMS

The aim of our experiment is to evoke an epileptic activity in rat hippocampal slices *in vitro* in our setup which is required for imaging techniques and NO detection in following researches and determination a role of NO in disorders of nervous system. We want to evoke this activity in following five experimental models: low-Mg^{2+}, high-K^{+}, Carbachol, Bicuculline and 4-AP model.

3.2 HYPOTHESIS

We assume that epileptic activity will be evoked in all experimental models (low-Mg^{2+}, high-K^{+}, Carbachol, Bicuculline and 4-AP) in our setup because of previous success of other investigators in these models mentioned in their reviews and because of well-prepared laboratory equipment.

4. METHODS

4.1 CHARACTERIZATION OF THE GROUP OF ANIMALS

Experiments were performed on slices prepared from adult male Wistar albino rats (100-180g). Rats were housed in a controlled environment (temperature 22 ± 1 °C, humidity 50-60%, lights on 06:00-18:00 h) with free access to food and water. Experiments were approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic. Animal care and experimental procedures were conducted in accordance with the guidelines of the European Community Council directives 86/609/EEC.

4.2 SLICE PREPARATION AND MAINTENANCE

For the purpose of this study we have used live rat brain tissue slices. Rats were deeply anaesthetized with ether and decapitated. Brain was rapidly removed from the scull, cut in oxygenated (95%) and cooled (-0, 5 °C) artificial cerebrospinal fluid (ACSF) using vibratome in coronal slices of 400μm of thickness. The slices contained both, the somatosensory cortex and the hippocampus, were transferred immediately after the preparation to a holding chamber filled with ACSF and oxygenated; they were placed on the chamber bottom on a lens paper for easier later manipulation. After 120 minutes recovery they were submerged into recording chamber through which oxygenated ACSF was perfused (5 ml/min) using a peristaltic pump (Minipuls 3 - GILSON) and perfused in stable temperature ± 36 °C. The temperature was maintained by Temperature Controller (SuperTech). The composition of the perfusion solution was (in mM): 126 NaCl; 26 $NaHCO_3$; 1.8 KCl; 1.25 KH_2PO_4; 1.3 $MgSO_4$; 2.4 $CaCl_2$; 10 glucose.

The recordings were made in the submerged recording chamber. Slice was fixed in the chamber on the mesh glued and the ACSF was perfused in mentioned speed above and below the slice and then drained away. Stimulating and detection electrode

were fixed as mentioned below with the eye-control in microscope and microscope imaging on PC with cooled 12-bit CCD-camera (RETIGA2000R).

4.3 ELECTROPHYSIOLOGICAL SYNAPTIC ACTIVATION

Synaptic activation of the tissue was made with bipolar stimulating electrode placed in hillus of the dentate gyrus of hippocampus (Mossy fibers). The stimulating electrode is made from double-chamber glass tube with double silver wire inside. For field potentials recording extracellular glass microelectrodes (8-10 M Ω) filled with 4M NaCl was used and placed in pyramidal layer of CA1 region (Fig. 2.3). The detection electrode is made from glass tube (theta tube) filled with NaCl fluid (as mentioned above) and chromed silver wire. 10 minutes after the replacement of the slice into the recording chamber, single shock stimulation with supramaximal stimulus was applied and field responses were recorded. The very first stimulation was done for determination of the functionality of stimulating electrode, to find the place in the tissue suitable for recordings and for the functionality of the whole slice.

Stimulation was made according to stimulation protocol by paired pulse (p) at 125 ms with isolated pulse stimulator (MODEL 2100, A-M Systems). We stimulated the tissue every 5 minutes.

For the purpose of our study we changed a concentration of perfused ACSF. In every model we changed a concentration of ACSF depending on the characteristics of model which we wanted to evoke. At first, every slice was washed out with regular ACSF and then changed for that one with changed concentration. It must be oxygenated at least 5 min in advance before perfused and stored in the stable temperature. In the case of models based on extracellular ionic changes we decreased or increased ionic concentration in ACSF. And in the case of models with pharmaceutical effects we added the chemical into perfused ACSF with the syringe. We used the concentrations resulting from the empirical and mentioned in reviews of previous researches.

All these changes were written down into the protocol and the spontaneous activity was expected.

4.4 DATA RECORDINGS AND ANALYSIS

Data recordings were made on PC in the program Spike 2 suitable for interpretation of the length of data, duration of evoked activity, amplitude, frequency and characteristics of evoked discharges. Data were stored on computer hard disc and analyzed off-line.

For detailed data evaluation we used the characterization of paired-pulse stimulation and its response (latency, amplitude, paired pulse index and Coast Line Index – CLI) in every experimental model of our research. Recording data were transferred into Microsoft Excel program and then analyzed.

5. RESULTS

We evaluated eight hippocampal slices with the following results. We haven't been able to elicit tonic clonic seizures in our setup that is the reason why we have devoted all our time to electrophysiological recordings. The only model whose activity resembled to seizure activity was the high-K^+ model (Fig. 5.15 and 5.16).

With low-Mg^{2+} (Fig. 5.17), high-K^+ 10mM (Fig. 5.15 and 5.16), Bicuculline in combination with high K^+ (7mM KCl) (Fig. 5.18 and 5.19), 4-Aminopyridine (5.20) and except for Carbachol treated slices, we were able to elicit epileptic discharges.

We used paired pulse protocol with supramaximal intensity stimulation to assess changes in the activity of the slices and to evaluate the alteration in the excitability of the tissue (examples of this stimulation are imaged in Fig. 5.5 - 5.14).

As a comparison of these paired pulse stimulations we used a measurement of latency (in seconds) and amplitude (in milivolts). For this purpose we marked the line with following indicators (Fig. 5.1) of population spike - popspike (beginning of stimulation, early EPSP - excitatory postsynaptic potential, postsynaptic spike, late EPSP) and then we calculated a paired pulse index (PPI):

PPI = amplitude popspike2/amplitude popspike1

The number of this index indicates a presence of potentiation (PPI > 1) or depression (PPI < 1) in evoked responses of each pulse in paired pulse stimulation.

Then we calculated Coast Line Index (CLI) for each pulse (CLI1 and CLI2) in paired pulse stimulation again.

CLI = longitude of the recording line/0.01

This index represents a response characterization on impulse, respectively how much the recording line oscillates in time (in this case in 1ms), measured from the

postsynaptic spike indicator (see Fig. 5.1). Then we can imagine the excitability of stimulated tissue, the higher number the more excitable tissue (for results see Fig. 5.2 and 5.3).

Finally, we compared four slices in one day (2. 4. 2010) made in 4-AP model. It compares CLI index, respectively CLI2 in regular ACSF at the beginning of recordings and CLI2 in 250μM 4-AP model. We evaluated the results in percentage (how does this value increase or increase compared to CLI1 (see Fig. 5.4).

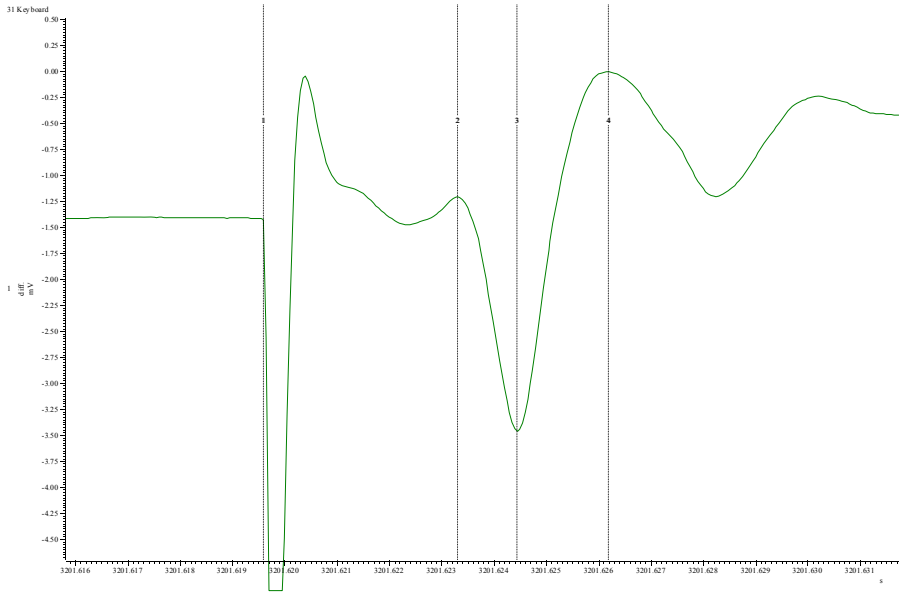


Figure 5.1: Indicators of evoked response (from left, 1 - stimulation, 2 - early EPSP, 3 - postsynaptic spike, 4 - late EPSP)

Figure 5.2: Data collection (1pop = latency of population spike in 1st pulse; 2pop = latency of population spike in 2nd pulse)

Date/slice/time (s)	Model	1pop (s)	1popspike (mV)	2pop (s)	2popspike (mV)
020410/c/134	ACSF	0.00547	0.462112425	0.00578	0.7345963
020410/c/3200	250uM 4-AP	0.0051	3.208618199	0.0048	3.638916
020410/c/4520	ACSF wash	0.0044	2.135162365	0.0047	1.4019394
020410/c/4513	ACSF wash	0.0045	1.370353705	0.004325	1.4542389
020410/b/573	ACSF	0.0045	0.463447575	0.00415	0.6104279
020410/b/1621	250uM 4-AP	0.0053	1.259460465	0.0049	0.7180023
020410/a/241	ACSF	0.00945	0.396690369	0.00947	0.4853439
020410/a/3791	250uM 4-AP	0.0046	1.116447466	0.0045	1.0322571
020410/d/46	ACSF	0.004775	0.29312134	0.004625	0.3103638
020410/d/2267	250uM 4-AP	0.005	0.918998705	0.0046	0.6985856
150210/a/830	low Mg	0.00778	0.2525711	0.01065	0.3694916
150210/a/1410	ACSF	0.0031	0.293083155	0.0058	0.3701401
150210/a/1666	13uM Bicuculline	0.0031	0.341377265	0.003	0.2521133
150210/a/3115	33uM Bicuculline	0.0033	0.32867432	0.0031	0.2455139
150210/c/710	ACSF	0.0065	0.288391109	0.00635	0.365448
150210/c/1307	30uM Bicuculline	0.0069	0.554161075	0.0075	0.6229019
150210/c/1930	30uM Bic + 200uM Carbachol	0.001	0.909156815	0.0006	0.0971603
150210/c/2064	low Mg	0.0034	0.26443479	0.0034	0.3070831
150210/c/4678	wash ACSF	0.0008	0.6487656	0.0009	0.6465149
150210/b/355	ACSF	0.0047	0.791091917	0.00438	0.8278275
150210/b/1303	50uM Bicuculline	0.004	1.13296509	0.0038	1.3227463
150210/b/2553	low Mg	0.0027	1.38557432	0.0031	1.0235214
150210/b/3455	ACSF wash	0.0036	1.31244659	0.0035	1.4216614
231009/a/52	ACSF	0.0045	1.370353705	0.004325	1.4542389
231009/a/695	10mM KCl	0.00363	0.0725937	0.00725	0.0540542
231009/a/4711	ACSF wash	0.0044	0.90351105	0.0044	0.9984971

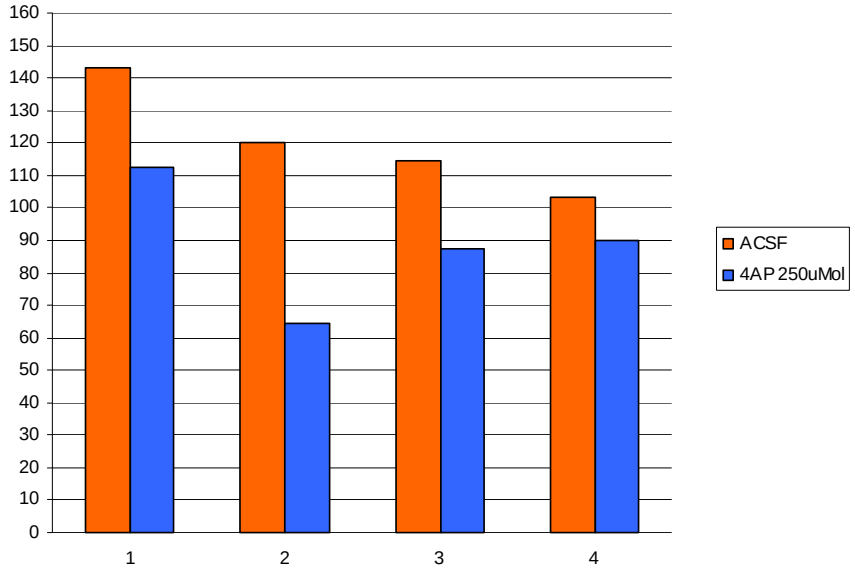
Note: 1-2pop = latency of population spike in 1st – 2nd pulse; 1-2popspike = amplitude in 1st – 2nd pulse

Figure 5.3: Data evaluation (paired pulse index, Coast Line index)

Date/slice/time (s)	Model	Paired pulse index	CLI1	CLI2
020410/c/134	ACSF	1.589648352	163.8105	234.5124
020410/c/3200	250uM 4-AP	1.13410688	864.5574	971.3748
020410/c/4520	ACSF wash	0.65659616	472.2708	553.7368
020410/c/4513	ACSF wash	1.061214272	515.2155	621.4124
020410/b/573	ACSF	1.317145429	212.4151	255.7017
020410/b/1621	250uM 4-AP	0.570087212	558.9084	358.7042
020410/a/241	ACSF	1.223483023	124.975	143.088
020410/a/3791	250uM 4-AP	0.924590841	491.0737	430.4463
020410/d/46	ACSF	1.058823506	159.0301	164.4512
020410/d/2267	250uM 4-AP	0.760159455	280.4199	252.2142
150210/a/830	low Mg	1.462921134	648.915	200.3217
150210/a/1410	ACSF	1.262918266	355.2399	192.1733
150210/a/1666	13uM Bicuculline	0.738518264	189.1159	367.1251
150210/a/3115	33uM Bicuculline	0.746982347	232.4215	299.7635
150210/c/710	ACSF	1.267195777	196.3368	322.1121
150210/c/1307	30uM Bicuculline	1.124044864	316.6129	642.2662
150210/c/1930	30uM Bic + 200uM Carbachol	0.106868621	293.8525	437.2391
150210/c/2064	low Mg	1.16128101	327.3163	681.7322
150210/c/4678.08	wash ACSF	0.996530796	648.43	530.8557
150210/b/355	ACSF	1.046436498	339.1489	624.5852
150210/b/1303	50uM Bicuculline	1.167508436	633.2958	255.7017
150210/b/2553	low Mg	0.738698325	654.2572	358.7042
150210/b/3455	ACSF wash	1.083214662	610.6081	143.088
231009/a/52	ACSF	1.061214272	515.2155	621.4124
231009/a/695	10mM KCl	0.744613513	362.7661	624.0694
231009/a/4711	ACSF wash	1.105129871	850.6203	887.6747

Note: CLI1 = 1st pulse; CLI2=2nd pulse)

Figure 5.4: Comparison of CLI1 a CLI2 in regular ACSF a 4-AP model in all experimental slice in 2. 4. 2010 (increase or decrease of CLI2 in the 2nd pulse in percentage) 100% was set as the popspike of the first response



Note 1: **1** - experiment 020410/slice c/, **2** - experiment 020410/slice b, **3** - experiment 020410/slice a, **4** - 020410/slice d

Note 2: In the first slice, we were able to evoke epileptic discharges, in the followings not, as we can see the values of the second stimulus response increased (potentiation) in the first slice and decrease in the other treated slices, which will be further discussed.

PAIRED PULSE STIMULATION

Following list includes the imagings of paired pulse stimulation and its responses. For imagination, it shows one example of every type in concentration of ACSF in certain recording situation (regular ACSF, ACSF wash - after using a pretreatment, low Mg^{2+}, high K^{+}, Bicuculline, Carbachol , 4-AP).

Figure 5.5: Example of paired pulse stimulation response, supramaximal stimulation intensity, conditions: **Regular ACSF** , 15. 2. 2010, slice b, 355s

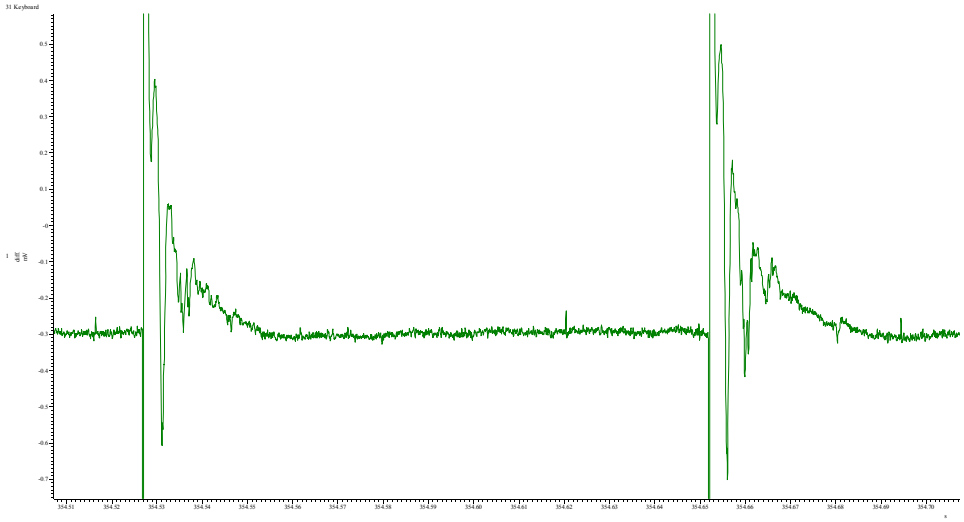


Figure 5.6: Example of paired pulse stimulation response, supramaximal stimulation intensity, conditions: **ACSF wash** , 23. 10. 2009, slice a, 4711s

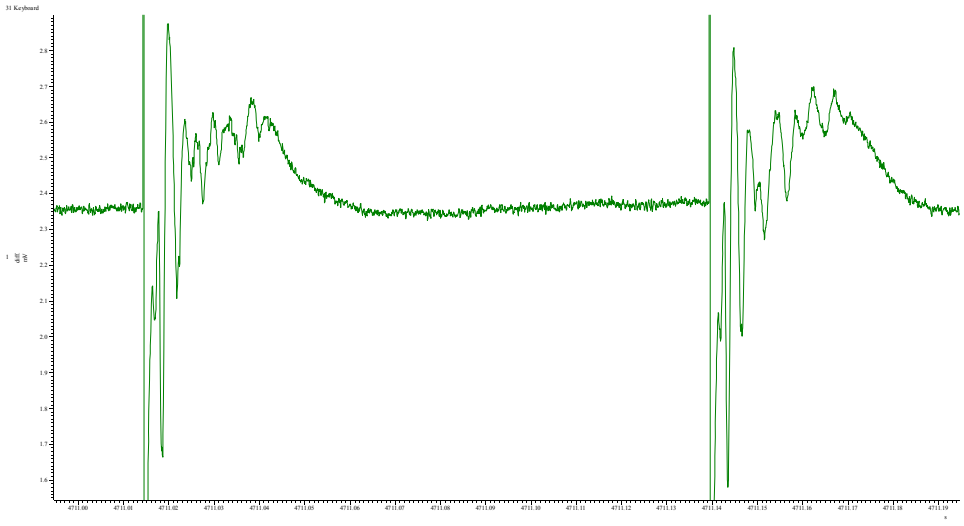


Figure 5.7: Example of paired pulse stimulation response, supramaximal stimulation intensity, conditions: **Low Mg^{2+}**, 15. 2. 2010, slice a, 830s

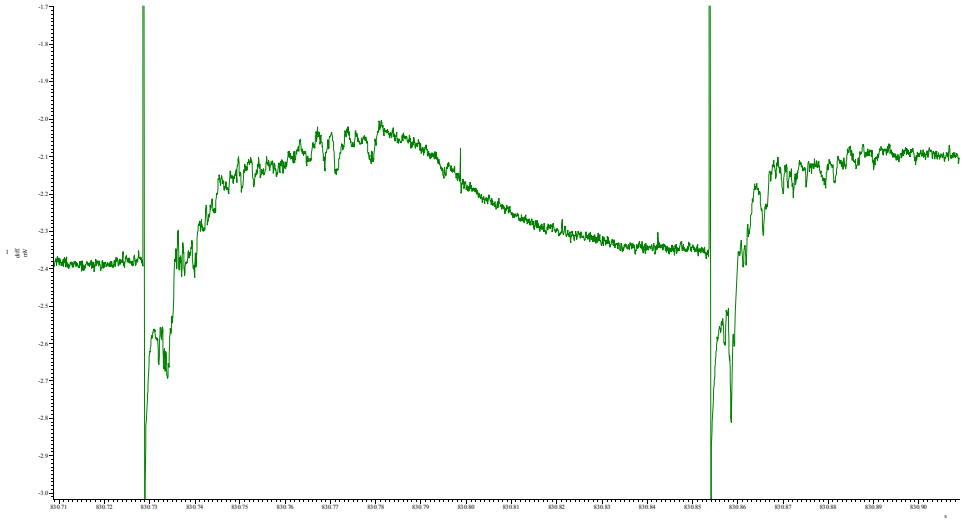


Figure 5.8: Example of paired pulse stimulation response, supramaximal stimulation intensity, conditions: **4-AP** , 2. 4. 2010, slice c, 3200s

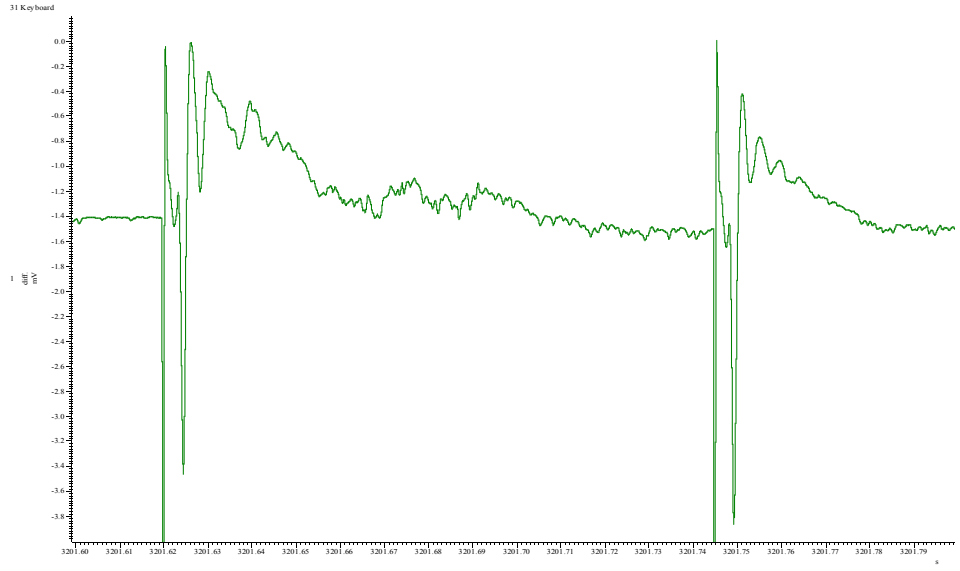


Figure 5.9: Example of paired pulse stimulation response, supramaximal stimulation intensity, conditions: **High K^+ (10mM KCl)**, 23. 10. 2009, slice a, 695 s

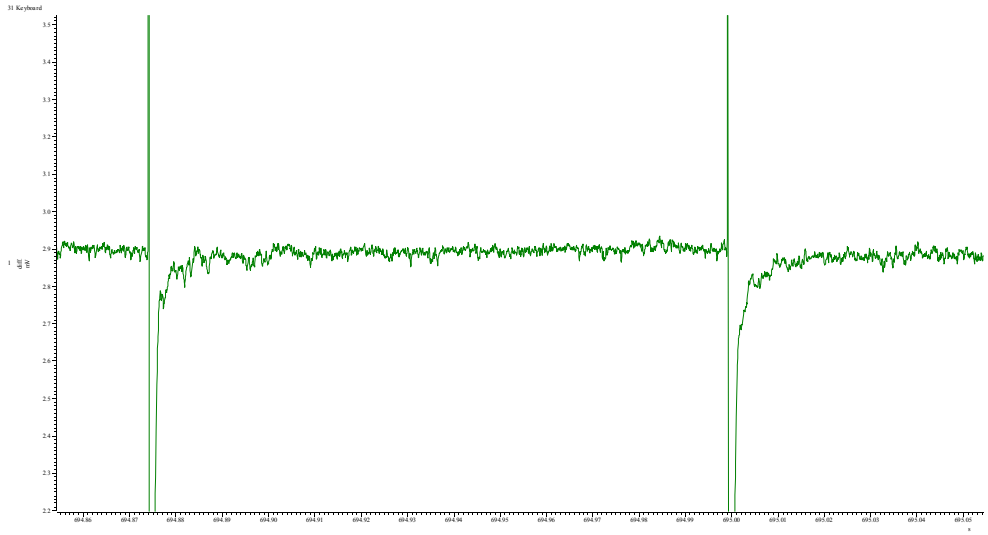


Figure 5.10: Example of paired pulse stimulation response, supramaximal stimulation intensity, conditions: **30µM Bicuculline + 200µM Carbachol**, 15. 2. 2010, slice c, 1930s

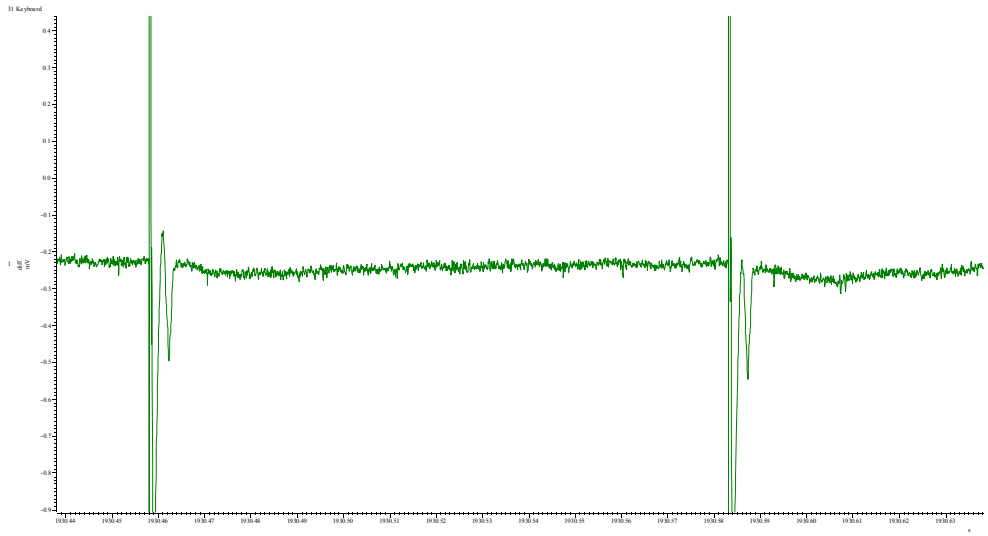


Figure 5.11: Example of paired pulse stimulation response, supramaximal stimulation intensity, conditions: **13µM Bicuculline**, 15. 2. 2010, slice a, 1666s

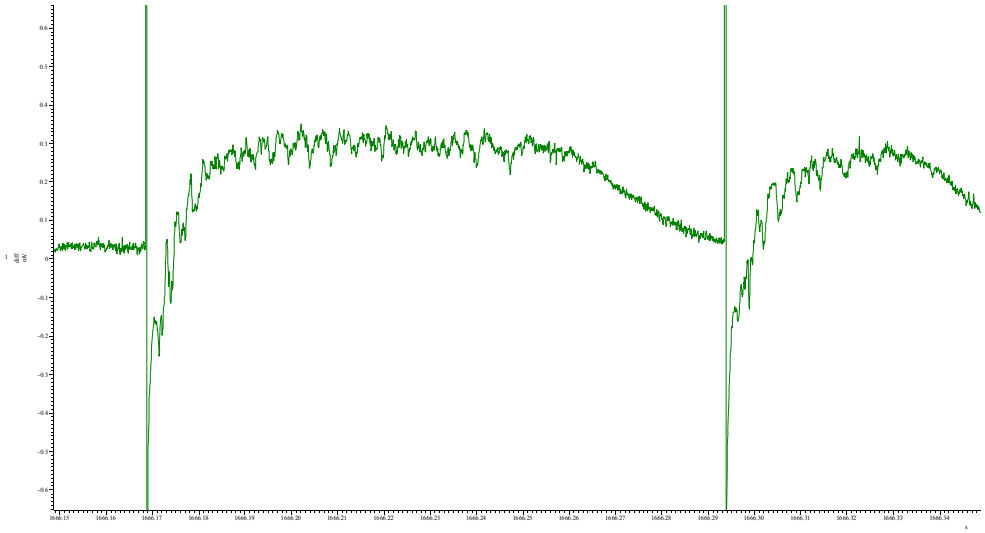


Figure 5.12: Example of paired pulse stimulation response, supramaximal stimulation intensity, conditions: **33µM Bicuculline**, 15. 2., slice a, 3115s

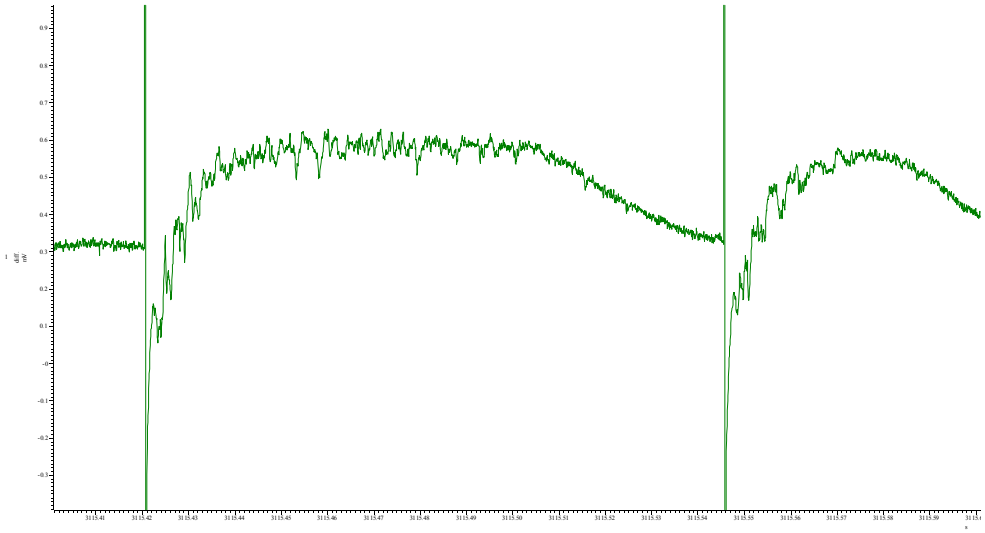


Figure 5.13: Example of paired pulse stimulation response, supramaximal stimulation intensity, conditions: **30µM Bicuculline**, 15. 2., slice c, 1307s

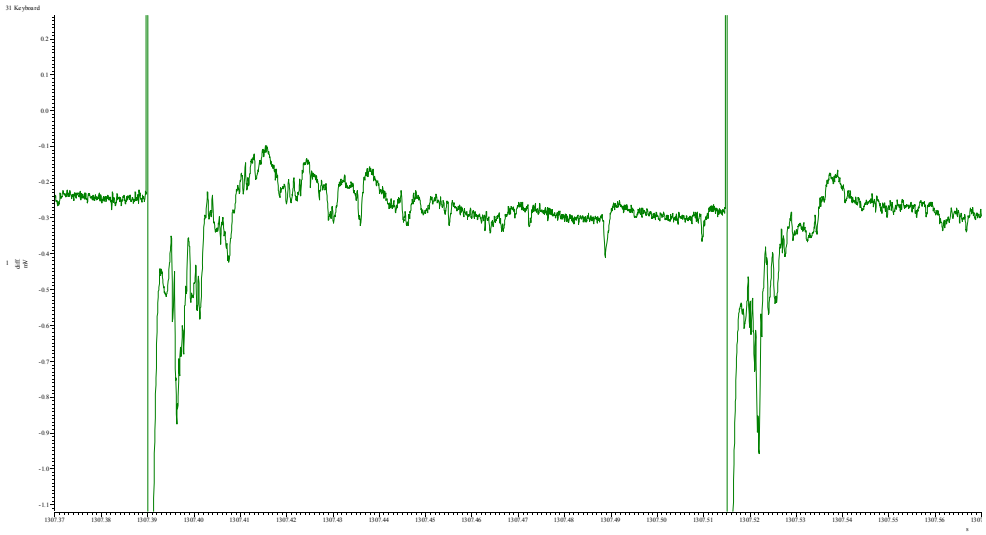
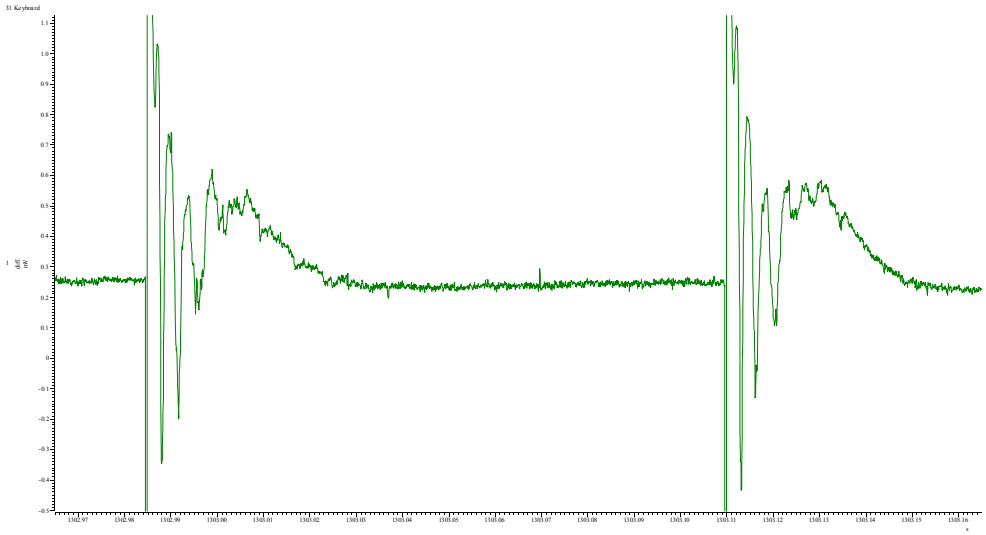


Figure 5.14: Example of paired pulse stimulation response, supramaximal stimulation intensity, conditions: **50µM Bicuculline**, 15. 2., slice b, 1303s



EPILEPTIC DISCHARGES

Following list includes the examples of discharges which were successfully evoked in four experimental models as we mentioned in results.

Figure 5.15: High K^+ (10mM KCl) - seizure like activity 23. 10. 2010, slice a

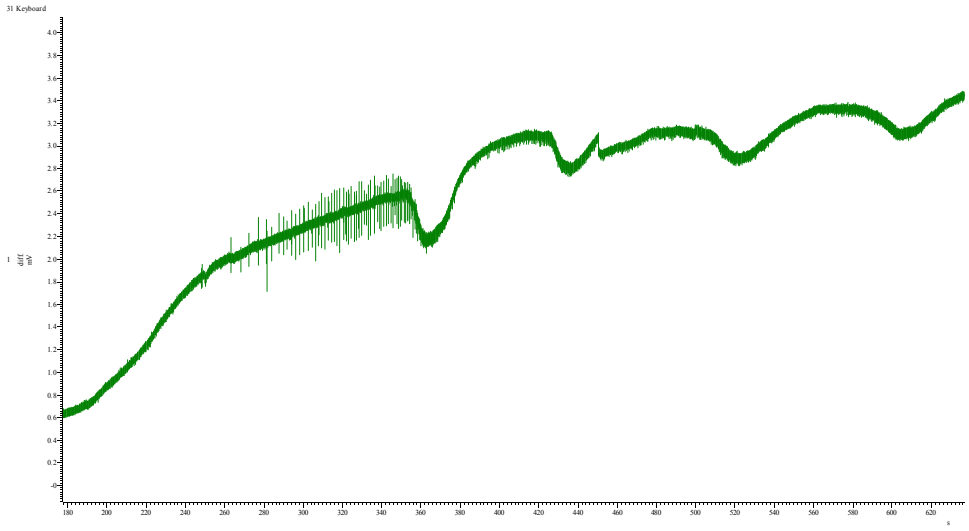


Figure 5.16: High K^+ (10mM KCl) - seizure like activity (zoom +) 23. 10. 2010, slice a

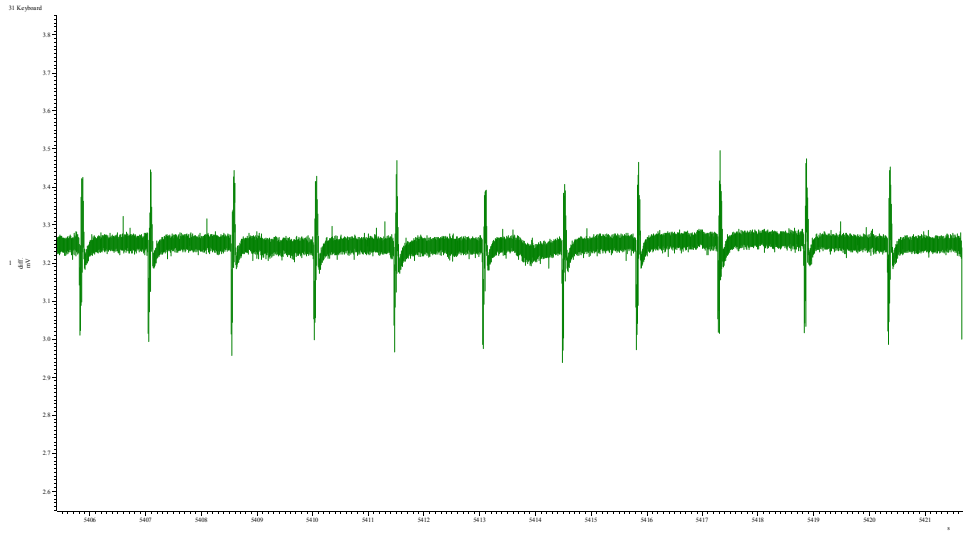


Figure 5.17: Low-Mg^{2+} (zero Mg^{2+}), 15. 2. 2010, slice a

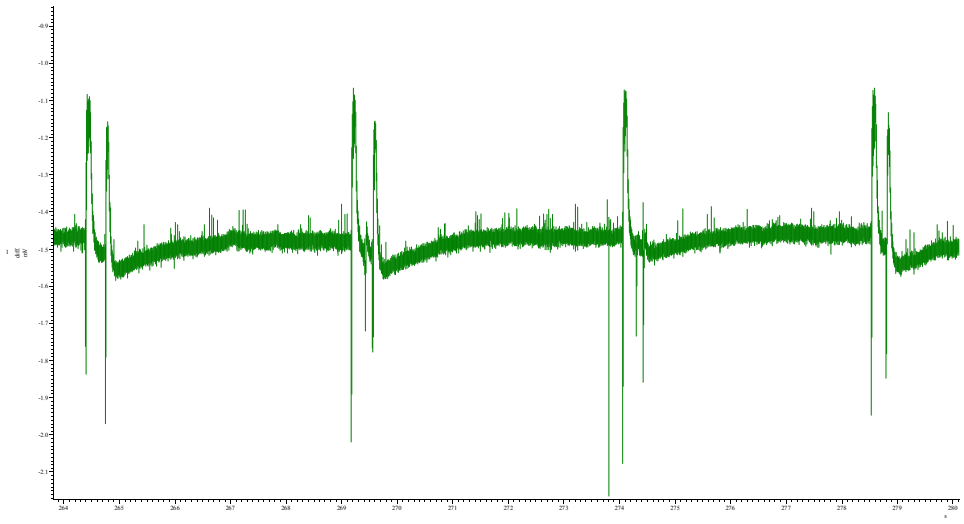


Figure 5.18: Bicuculline 13μM in combination with high K^{+} 7mM and after pretreatment with zero Mg^{2+}, 15. 2. 2010, slice a

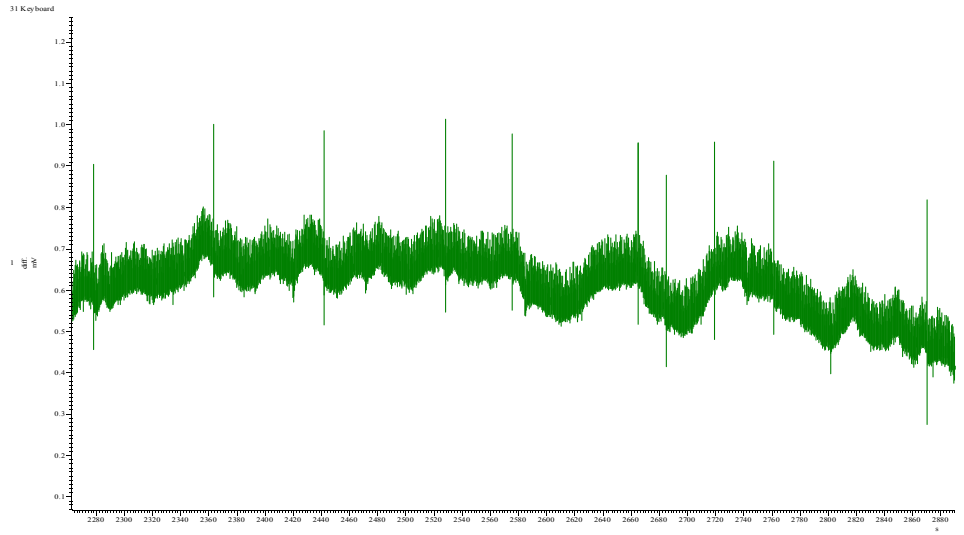


Figure 5.19: Bicuculline 13μM in combination with high K^+ 7mM and after pretreatment with zero Mg^{2+} (zoom +), 15. 2. 2010, slice a

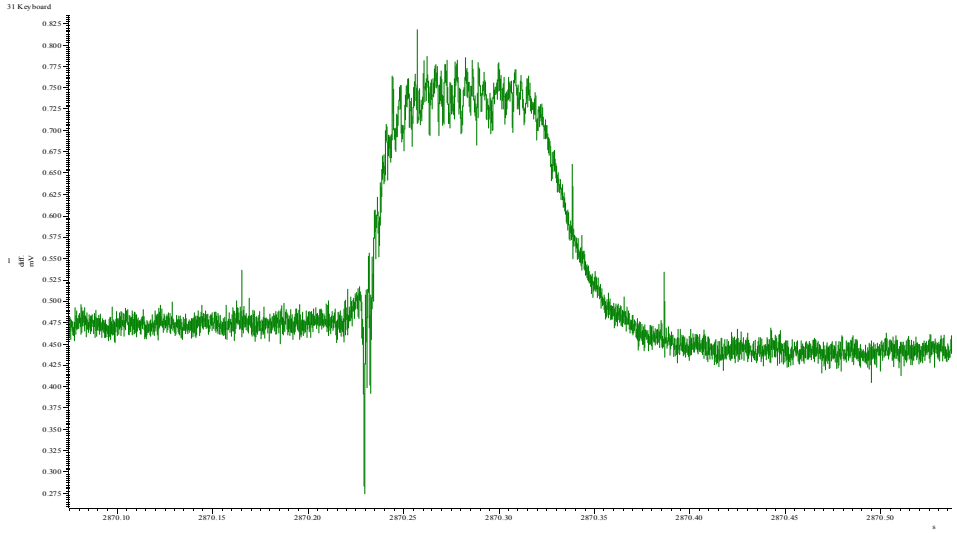
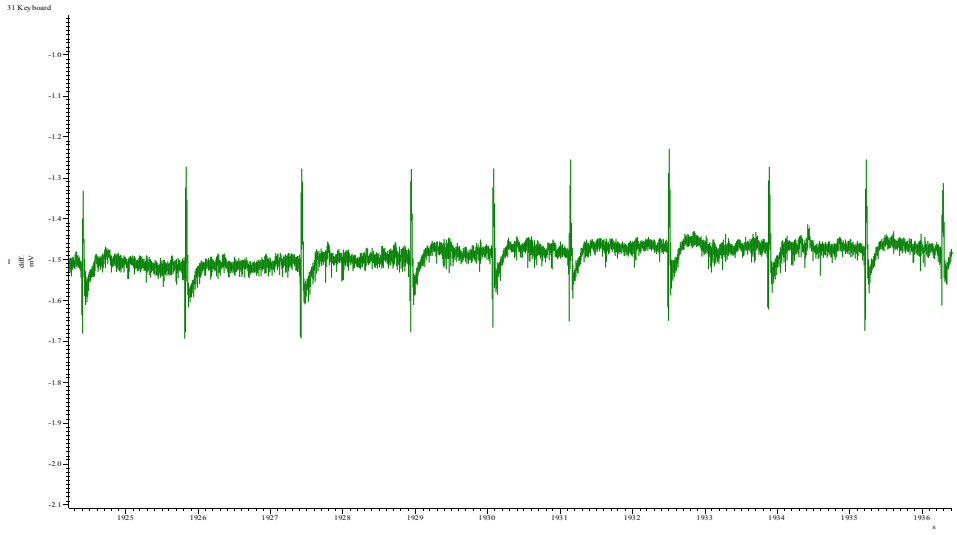


Figure 5.20: 4-AP (250μM), 2. 4. 2010, slice c



6. DISSCUSION

6.1 DISSCUSION - METHODS

We used a submerged type of chamber for maintenance of rat hippocampal slices and for recordings of electric activity in this tissue.

When using a submerged chamber, it brings some advantages for experiment because specifications used for slice maintenance remind more *in vivo* conditions; there is no gaseous atmosphere too and the cells are in direct contact and surrounded by liquid. And even more, this chamber is necessary for ability to perform the imaging techniques.

But simulation of *in vitro* conditions in this chamber brings many difficulties too because the cells definitely need a sufficient amount of oxygen supply and other substances for their nutrition such as glucose and it has to maintained in the constant ionic environment so that is the reason why ACSF must flow in adequate speed (5ml/min in our setup). This fast overflow of solution may cause a movement of the slice in the chamber and these movements and the noise resulting from a permanent flow of ACSF could disturb the detection of signal and transfer these noises into the recording data and it could be even harmful for the slice.

Unfortunately, this type of maintenance and recording of tissue still interferes with some crucial differences compared to real organism such a slower washout of the ions from the extracellular space and the difference in oxygen availability that could cause apparent discrepancies in the assessment of cell's function and its reaction to the stimulation. Oxygen is further delivered only through the diffusion in this non-vascular tissue and does not react on the energy demand via increase in blood flow which would normally occur *in vivo* after increased tissue demands.

Another important issue of experiment is the temperature at which is the tissue maintained because membrane receptors open and get activated only in certain temperature and SLE is very difficult to induce at temperatures below 33°C. The higher

temperature facilitated opening of for example NMDA receptors and the excitability is increased and it is well know that during febrile states especially in children, it can lead to so called febrile seizures (Hopkins et al., 1995).

Finally, it is necessary to pay high attention to preparation and cutting experimental tissue. This process must be done as quickly as possible, with sufficient oxygen supply and kept in stable temperature to maintain the viability of the slice and accomplished preciously to preserve maximal amount of intact neuronal connections. Even a little discrepancy in the angle of cutting and preparation can damage many connections and destroy the tissue. These conditions are therefore imperative for following manipulation within slices and correct recordings without any misunderstandings.

6.2 DISSCUSION - RESULTS

In vitro studies made on rat brain slices and slice cultures are suitable for the stimulation of electrical activity which is similar to seizure activity we detect *in vivo* in certain pathophysiological states. Our aim in this study was to set several models of epileptic tonic-clonic activity *in vitro* that would be suitable for the detection of NO production in condition during states of abnormal seizure activity.

In our conditions we were so far not able to find a good model for such detection. We have picked up several models that disrupt normal activity by increasing the excitability and can lead and often lead to epileptic discharges.

The most reliable models of seizure activity *in vitro* were set especially while working on tissue cultures. Our situation is little bit more difficult because of the fact we are working with acute slices of the hippocampus where the neurons are damaged during cutting of the tissue and eliciting seizure activity is therefore harder considering damaged cells and worse connectivity.

Finally we were not able to elicit seizure like event with tonic-clonic seizures in any model that was available. That is the reason why we used for the discussion of the results the evaluation of paired pulse stimulation protocol to at least access the changes

in the excitability. Further we evaluated the Coast Line Index to have the other criteria mainly for the cases where the shape of the evoked response changed and where the length of the whole curve was longer.

The only model whose activity resembled to seizure activity was the high potassium model (Fig 5.15 and 5.16), where we were able to elicit discharges with the concentration of 10mM and depolarization of the tissue which might be hold as a seizure. The increased potassium level occurs at seizure activity (around 8mM (Leschinger et al., 1993) and increases the excitability of neurons. We were still not entirely satisfied with this model because neither we could elevate the level of potassium concentration or sustain this lever for longer time because 10mM concentration was rather high and such elevated level of potassium could possibly lead to status called spreading depression, which is long lasting wave of depolarization of the tissue and this would prevent the tissue from activity. The suitable model would be to start this activity with 10mM K^+ and continued with 7 - 8mM but with such concentration we were not able to sustain the activity. Paired pulse measurement indicates the depression in response and CLI higher excitability of the tissue.

For the other model we tried Bicuculline which acts through $GABA_A$ receptors and inhibits the GABA inhibition of interneurons and therefore increases the excitability. With our model, we were not that successful in eliciting neither the seizures nor epileptic discharges. The only slice where we were able to get epileptic discharges (13 and 33μM) we would be able to describe as a bursting activity (Fig. 5.18 and 5.19) were the slices pretreated with zero Mg^{2+} model (tonic activation of NMDA receptors). We conclude that the tonic activation of the slice is able to change the properties of the nervous tissue and start epileptogenesis and might be of our further interest while searching for the other new model. Evaluation of paired pulse response (Fig. 5.11 for 13μM Bicuculline, Fig. 5.12 for 33μM Bicuculline, Fig. 5.13 for 30μM Bicuculline and Fig. 5.14 for 50μM Bicuculline) and CLI displays higher excitability in the tissue, except of model with 50μM Bicuculline where the tissue seemed to be over stimulated because of high concentration of Bicuculline.

4AP was used in the concentration of 250μM according to previous protocol (Schuchmann et al., 1999). With this model, we were not able to elicit seizure like activity. In one slice of four (Fig. 5.20) there was interictal activity which would not be

probably sufficient activity for the detection of the NO production. The evaluation of paired pulse stimulation and Coast Line Indexes showed increased excitability in terms of regular ACSF concentration but not in 4-AP model; and paired pulse index evaluates the potentiation in ACSF in every slice and depression in 3 of 4 slices treated with 4-AP (Fig. 5.3). Comparison of CLI1 and CLI2 both terms (ACSF and 4-AP) in graph in percentage evaluate that there is an increase of excitability in ACSF but decrease in 4-AP (Fig. 5.4).

The other idea was to use Carbachol in the concentration of 200μM to influence cholinergic pathways in combination of 33μM Bicuculline to start the seizure (Fig. 5.10). But the effect was opposite and we inhibited the activity, which lead to the decrease of the pair pulse response and decrease in the latency of the second response (Fig. 5.2 and 5.3).

The final model I am going to mentioned is zero Mg^{2+}. Firstly we wanted to avoid the model of zero Mg^{2+} because of the massive tonic activation of NMDA receptors that is not natural and wouldn't be possible to be simulated in vivo. We evoked epileptic discharges in this model (Fig. 5.17). Unfortunately even with this model, we were not able to elicit tonic-clonic seizures, which might lead to assumption that in our conditions we are not able to elicit this kind of activity at all and so we should think about some changes in our experimental conditions such as ACSF flow, temperature, position of slice in the chamber or cutting slices.

7. CONCLUSIONS

We were not able to elicit tonic-clonic seizures that we would need for further detection of NO production. The only model whose activity resembled to seizure activity was the high-K^+ model (10mM).

We were able to elicit epileptic discharges during low-Mg^{2+} (zero Mg^{2+}), the model evoking the massive tonic activation of NMDA receptors; high-K^+ (10mM), the only model whose activity resembled to seizure activity; Bicuculline (13μM and 33μM) in combination with high K^+ (7mM KCl) and pretreatment with zero Mg^{2+}, the model based on inhibition of GABA which results in epileptic discharges only in one slice and with the characteristics of bursting activity; and 4-Aminopyridine (250μM), the model in which we evoked interictal activity but probably not sufficient for the detection of the NO production.

We suggest that we will have to change the measuring conditions of the tissue for further success in eliciting the epileptiform activity in rat hippocampal slice *in vitro* regarding different ACSF flow or temperature or position of the tissue in the chamber or to play around with the cutting of the hippocampal slices to improve the conditions for slice.

8. ABBREVIATIONS

ACSF - artificial cerebrospinal fluid

AMPA - amino-3-hydroxy-5-methylisoxazolepropionic acid

ADP – adenosine diphosphate

ATP - adenosine triphosphate

BOLD fMRI - functional magnetic resonance imaging

Ca - Calcium

cGMP - cyclic guanosine monophosphate

CNS - central nervous system

CSF - cerebrospinal fluid

DG - dentate gyrus

EC - entorhinal cortex

EEG - electroencephalography

GABA - γ-aminobutyric acid

K - Potassium

KA - kainic acid

L-NAME - N (G)-nitro-L- arginine methyl ester (hydrochloride)

L-PIA - L-phenyl isopropyl adenosine

LTD - long-term depression

LTP - long-term potentiation

Mg - magnesium

MS - Multiple Sclerosis

NADH - nicotinamide adenine dinucleotide

NADPH - α-nicotinamide adenine dinucleotide phosphate

NMDA - N-methyl-D-aspartate

NO - nitric oxide

NOS - nitric oxide synthase

eNOS - endothelial nitric oxide synthase

iNOS - inducible nitric oxide synthase

nNOS - neuronal nitric oxide synthase

PDS - paroxysmal depolarization shift

PTE - posttraumatic epilepsy

ROI - region of interest

SD - spreading depression

SLE - seizure-like event

T - threshold

4-AP - 4-Aminopyridine

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Images

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